Universidade de Aveiro Departamento de Biologia
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António Manuel de Andrade Lapa Amaro

FERRAMENTAS PARA AVALIAÇÃO ECOTOXICOLÓGICA DE SOLOS CONTENDO BIOCHAR

OPTIMISED TOOLS FOR TOXICITY ASSESSMENT OF BIOCHAR-AMENDED SOILS

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, ramo toxicologia e ecotoxicologia, realizada sob a orientação científica da Doutora Ana Catarina Bastos, Investigadora de Pós-Doutoramento do Departamento de Biologia e CESAM da Universidade de Aveiro e co-orientação da Doutora Susana Patrícia Mendes Loureiro, Investigadora Auxiliar do CESAM e Departamento de Biologia da Universidade de Aveiro e do Doutor Miguel João Gonçalves dos Santos, Investigador de Pós-Doutoramento do Departamento de Biologia e CESAM da Universidade de Aveiro.

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O juri

Presidente / President	Professora Doutora Maria Adelaide de Pinho Almeida Departamento de Biologia da Universidade de Aveiro
Vogal – Arguente / Examinor	Doutor Tiago Manuel Ferreira Natal da Luz Investigador Pós-Doutoramento, Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade de Coimbra
Vogal – Orientador / Supervisor	Doutora Ana Catarina Gomes Marcelo Bastos Investigador Pós-Doutoramento, Departamento de Biologia da Universidade de Aveiro

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Palavras-chave biochar, solos, bioensaio, mesocosmos, ecotoxicologia.

Resumo

O crescente interesse da aplicação de biochar em grande escala em solos, seja para melhorar o rendimento das culturas, como uma ferramenta para o sequestro de carbono ou para a substituição de fertilizantes inorgânicos, realça a urgência de uma avaliação eficaz dos potenciais efeitos negativos sobre a biota do solo, assim como nos processos e funções do solo que medeiam. Enquanto a ecotoxicologia do biochar está a tornar-se progressivamente mais importante, os estudos que se concentram nos efeitos do biochar em espécies terrestres representativas são poucos e ainda sem relevância prática a nível ambiental e ecológico. Este estudo centrou-se na adaptação e otimização de uma série de ferramentas ecotoxicológicos e ecológicos (que foram criados ou padronizado para solos contaminados), a fim de testar a sua adequabilidade na avaliação do potencial tóxico de solo alterados com biochar.

Em primeiro lugar, foi testada a adequabilidade de testes de evitamento em invertebrados (utilizando minhocas, colêmbolos e isópodes) na avaliação do potencial toxico de solos enriquecidos com biochar de origem vegetal, quer isoladamente quer em combinação com adubo tradicional, ao longo de um período de cinco meses, num teste de campo realizado na Estação Vitivinícola da Bairrada. No entanto, existe uma crescente necessidade de mais condições de representatividade em testes, que tenham em conta períodos de tempo mais longos e maior variabilidade ambiental (por exemplo, a umidade do solo, temperatura) e ecológica (ex: interacções entre organismos teste coexistentes e sua distribuição vertical no solo). Desta forma, a segunda parte deste trabalho foca-se na optimização de uma metodologia, utilizando um modelo de ecossistema terrestre de pequena escala contendo minhocas e plantas, para se obter um estudo superior e mais representativo dos impactos potenciais de biochar produzido através de estrume em ecossistemas terrestres a taxas de aplicação propostas.

Os resultados sugerem que as respostas de comportamento de evitamento usando organismos invertebrados representativos podem ser usadas para avaliar o impacto do biochar de origem vegetal na biota do solo, num cenário real, ao longo de 5 meses. Isto pode ter implicações para complementar outras estratégias que tenham em vista a caracterização ou gestão das concentrações de referência de biochar a aplicar em solos, assim como na ajuda a escolher o tipo de biochar e taxas de aplicação de segurança. Além disso, a utilização de STEMs contendo minhocas e sementes de nabo expostos ao biochar produzido através de estrume sob condições mais representativas mostrou ser mais conservador quando comparada com os testes normalizados de apenas um teste e, portanto, pode ser adequado numa avaliação de nível superior do potencial tóxico de solos com biochar feito a partir de estrume. Estudos como o presente poderão ser uma contribuição importante para o estabelecimento de metodologias adequadas de avaliação de risco de biochar e assim apoiar tanto no desenvolvimento contínuo dos sistemas de normalização de biochar e também no desenvolvimento de regulamentos biochar adequados.

Keywords biochar, soils, bioassays, mesocosms, ecotoxicology.

Abstract The growing interest in large-scale biochar application to soils, either for improving crop yield, as a tool for carbon sequestration or for replacing inorganic fertilizers, highlights the urgency for an effective evaluation of potential negative effects on soil biota, and the soil processes and functions that they mediate. While the field of biochar ecotoxicology is becoming more important, studies that focus on biochar effects on selected terrestrial species remain scattered and lacking environmental, ecological and practical relevance. This study focused on adapting and optimising a range of ecotoxicological and ecological tools (that have been established or standardized for contaminated soils), in order to test their suitability for evaluating the toxic potential of biochar-amended soils.

Firstly, it was tested the suitability of invertebrate avoidance behaviour assays (using earthworms, collembolans and isopods) to assess the potential toxicity of soils enriched with wood-biochar, alone and in combination with traditional compost, over a 5 month period, in a real field trial at the Estação Vitivinícola da Bairrada. Nevertheless, there is increasing need for more representative conditions in testing, that account for longer study durations and greater environmental (e.g. soil moisture, temperature) and ecological variation (e.g. interactions among co-existing test organisms and their vertical distribution in soil). The second part of this work therefore, focused on optimizing a methodology using Small-scale Terrestrial Ecosystem Models (STEMs) containing earthworms and plants, for higher-tier studying of the potential ecological impact of manure-biochar on terrestrial ecosystems, at reported application rates.

Results suggest that avoidance behaviour responses using representative invertebrates can be used for evaluating the impact of wood-biochar on soil biota, in a real case application, over 5 months. This can have implications for complementing other strategies for characterizing or

managing biochar field applications, such as help with the choice of biochar type and safe application rates. Further, the use of STEMs containing earthworms and turnip seeds exposed to manure biochar under more representative conditions, has shown to be more conservative when compared to the standardised single species test and therefore, may be adequate as higher-tier evaluation of the toxic potential of soils with manure biochar. Studies like the present can be an important contribution for establishing suitable biochar risk assessment methodologies and support both, on-going development of biochar standardization schemes and development of adequate biochar regulations.

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ANOVA	analysis of variance
C	carbon
CEC	cationic exchange capacity
EC	effective concentration
FW	fresh weight
G.	germination
IBI	International Biochar Initiative
ISO	International Standard Organization
N	nitrogen
NOM	natural organic matter
OECD	Organization for Economic Co-operation and Development
P	phosphorous
PAH	polycyclic aromatic hydrocarbon
S	sulphur
SL	shoot length
SOM	soil organic matter
STEM	Small-Scale Terrestrial Ecosystem
WHC	water holding capacity

1 General Introduction, Research Aims and Relevance

1.1 Biochar: its Nature and Concept

1.1.1 Historic Outlook and Notions

Soil properties are variable over time and from region to region due to a great number of biotic and abiotic factors, as well as their interactions, being dependent on geological, climatic and geographic characteristics. Soil physic, chemical and biological characteristics, including structure as well as biota abundance, activity and community composition, influences a large number of soil processes and functions, including water retention and nutrient cycling, and consequently soil productivity and plant fertility (Fanning and Fanning, 1989). Highly sorbent and porous soils (e.g. those with high clay content) have a natural inherent capacity to retain more water and nutrients than soils with a more sandy texture (Cornelissen et al., 2005a, Cornelissen et al., 2005b). These characteristics, which rely on the greater pore volume (i.e. surface area) and cationic exchange capacity (CEC), are beneficial to many soil processes and functions to different extents, such as water retention, biological activity and recycling of organic matter, immobilization/mineralization of soil contaminants, productivity and maintenance of water quality, overall linked to soil quality (Liang et al., 2006, Beesley et al., 2010). With the intent of exploiting soils in order to maximize their efficiency in fulfilling their functions, there have been various developments throughout history, with some success (e.g. compost) (Amlinger, 2007) while other strategies relies in the introduction of compounds as source of nutrients to enrich soils composition(e.g. fertilizers) (Tisdale et al., 1985, McGrath et al., 2003). However, the stability of the ecosystem can be threatened by anthropogenic activity that may alter soil conditions beyond what is sustainable and lead to deterioration or loss of soil function, since dynamics between mineral, organic and biological soil components are complex and sensitive.

Biochar, a type of charcoal (i.e. charred organic matter) that has been lately widely investigated, appears as a candidate for application to soils to promote soil quality and ecosystem function. Biochar is the carbon-based product of pyrolysis, an exothermic process employed for the production of biofuels (e.g. syngas), in which degradation of carbon-rich biomass occurs in the absence of oxygen. In this process, factors such as type of feedstock source and temperature are the main influencing factors for defining the properties of the resulting biochar, in terms of quantity, chemical composition and structural (e.g. atomic) arrangement. The difference between biochar and charcoal goes further than terminology and rely on their use and function, referring to biochar when it is intended to be applied to soils for performance improvement and charcoal when it is used as fuel. Further, charcoal is produced almost exclusively from wood, whereas biochar can have a variety of feedstock, often depending on the desired properties of the final product. This distinction is useful and important from the view of both soil science and environmental quality, as suggested by Verheijen et al. (2010).

Unlike biochar, charcoal formation occurs spontaneously in nature, where organic material is partially combusted, such as during wildfires, without being associated to any kind of controlled process, and as such, a certain amount of charcoal is present in soils around the globe. Soils with a considerable amount of charcoal were found in small pockets all around the Amazonian basin, one of the worldwide places with higher plant density and diversity. Similarly to other tropical soils worldwide, Amazonian soils are generally nutrient-poor and were unable to support crops, reason for which indigenous populations tried to improve crop production. These soils, were named “Terra Preta” (Black Earth in English) and became known for their higher fertility compared to neighboring soils, while being result of progressive accumulation and mixing of charcoal with rich organic anthropogenic substances derived from fishing, hunting and other human activities (Glaser and Birk, 2012, Glaser et al., 2001). Acknowledging the relationship between charcoal occurrence in soil and improved soil fertility is the foundation for the modern biochar concept. Thus, its application to agricultural soils, is foreseen to benefit soil characteristics, processes and functions (Lehmann et al., 2006, Lehmann and Rondon, 2006, Blackwell et al., 2009, Atkinson et al., 2010, Jeffery et al., 2011), while offering a mean for sequestering carbon (thus maybe helping to mitigate climate change when considered alongside other strategies) (Van ZwietenA et al., 2008, Sohi et al., 2009) and diminishing bioavailability of soil contaminants (Uchimiya et al., 2010, Beesley and Marmiroli, 2011, Beesley et al., 2011, Cao et al., 2011, Park et al., 2011, Jiang et al., 2012).

1.1.2 Major Biochar Attributes and Effects

Due to biochar physical and chemical characteristics, such as stable structure, owing to high content in aromatic carbon (C), high porosity resulting in large surface area, high cation exchange capacity (CEC) and chemically reactive surface and neutral-to-alkaline pH, it has become the subject of many investigations in science and environmental management. The molecular structure of biochar (such as the degree of aromaticity and arrangement of carbon) makes it physically stable and recalcitrant to physical, chemical and biological degradation in soil at atmospheric conditions. Contrary to the labile fresh organic residues that are incorporated into soil as part of traditional agricultural management (e.g. for improving soil structure and nutrient content), biochar can remain in soil for centuries or millennia. This endurance in soil allows biochar to slow down the carbon cycle and thus sequester the carbon locked by plants during photosynthesis, while maintains it during its long residence time. By diminishing the release of atmospheric carbon as well as of other greenhouse gas emissions from biological activity, such as nitrous oxide (N_2O) and methane (CH_4), it can contribute to mitigate climate change (Lehmann et al., 2006, Spokas et al., 2009).

The porous nature of biochar improves soil structure by increasing its surface area while diminishing its bulk density. A less dense soil has improved water dynamics (e.g. retention, drainage), increased aeration and gas exchange, allowing the roots to expand easier, and benefiting the locomotion of invertebrates, which as indirect consequence, leads to an overall increase in biological performance. In addition to the porous structure, biochar' high CEC and liming properties (due to high pH) can enhance the retention of nutrients and benefit productivity of acidic soils, respectively. Adding to the fact that fresh biochars can be an input source of nutrients in soil (particularly in the form of minerals), the result is augmented microbiological activity, leading to improved soil fertility and plant productivity, allowing a wider number of soil organisms to establish and prosper, and thus contributing to maintain biodiversity and ecological functions (Schwartz et al., 2006, Atkinson et al., 2010, Compant et al., 2010, Graber et al., 2010). Therefore, there is potential for biochar to lead to a positive overall impact on the ecosystem in the long-term, with improved cost/benefit when compared to other soil amendments, as long as the selection of a given biochar to a given application is performed adequately, and in the way that matches the requirements of the soil under consideration (Verheijen et al., 2012).

However, there are other motivations for biochar production and application, such as for enhancing immobilization of soil pollutants, due to combining an extensive and reactive (rich in functional groups) surface area, allows biochar to react with and adsorb a wide range of compounds, such as organic substances (ex. polycyclic aromatic hydrocarbons - PAHs) (Chiou and Kile, 1998, Cornelissen and Gustafsson, 2003, Koelmans et al., 2006, Cao et al., 2009, Cao et al., 2011) and metals (Beesley and Marmiroli, 2011, Park et al., 2011, Uchimiya et al., 2011, Jiang et al., 2012). These properties make biochar a product of great interest as a remediating agent, even more by the fact that it's both stable and recalcitrant, while in practice means an immobilization of pollutants during biochar's long lifetime (Zimmerman et al., 2004, Brändli et al., 2008, Cao et al., 2009, Beesley et al., 2010, Fagervold et al., 2010, Beesley and Marmiroli, 2011). The same principle could also be applied to organic wastes and water treatments, which currently use activated charcoals as a clean-up means, although at a higher cost. In addition, biochar production may also be a possible solution for reducing organic wastes and thus improving waste management, since various green wastes (e.g. forestry residues, fruit pulp, grain husks, peanut shells, olive stones, manures) can be seen as suitable biomass sources to be transformed through pyrolysis for biochar production (Verheijen et al., 2010, Lehmann et al., 2011).

1.2 Biochar-N: an Alternative in Management of Organic Wastes while Boosting Crop Yields

Biochars produced from organic wastes such as manure, have also shown effective as a potential alternative to inorganic N-fertilizers (N source and sink), at reduced costs while supporting waste management (Day et al., 2005, Lehmann and Joseph, 2009). Since organic wastes are rich in a vast number of nutrients (e.g. N, P, S), biochar obtained from this source of biomass can be a relevant alternative to traditional inorganic N-fertilizers. Furthermore, adding biochar into a soil previously sprayed with liquid manure has shown to decrease odor emissions from urea products (e.g. ammonia, NH_3), while enriching the soil with nitrogen, in forms that are bioavailable for plants (Schmidt, 2012). At the same time, biochar prevents the building-up of high concentrations of NH_3 which would become toxic to terrestrial and aquatic communities, although still allowing NH_3 to be converted into non-toxic metabolites. Ammonium (NH_4^+, formed by the protonation of ammonia) is one of these products that have an important role in the biogeochemical nitrogen cycle for supplying nitrogen to plants. The rate of conversion of toxic ammonia to ammonium products by biological activity can be increased in the presence of some types of microorganisms at low pH (Factura et al., 2010). Therefore, biochar post-treatment with lactic acid bacteria responsible for lactic acid fermentation (that has been used by humans in a wide range of fields), presents itself as an excellent option to eliminate toxic compounds and convert nitrogenous toxic into non-toxic and bioavailable forms (Schmidt, 2012).

1.3 Biochar contaminants and interactions with soil fauna

1.3.1 Total and bioavailable contaminants in biochar

The presence of contaminants in biochar is frequent, being metals and PAHs the most prominent. As previously explained, feedstock and pyrolysis conditions are determinant for biochar heterogeneity and behaviour, and as such, they also explain the presence of toxic substances in fresh biochar. For most biochar contaminants, it remains unknown how large is their bioavailable fraction once biochar is applied to soil, with adequate methodologies for quantification of bioavailable biochar contaminants not having yet been developed. Biochar production has drawn attention regarding the potential consequences and implications of such contaminants to environmental quality, even more if one considers its stability and recalcitrant behaviour in the environment. It is consensual that biowaste sources (e.g. animal manure, sewage sludge etc.) have higher levels of organic and metal contaminants than more traditional sources (e.g. wood, crop residues, and grass). For instance, Bridle and Pritchard (2004) found that biochar derived from sewage sludge had high level of metallic contaminants, such as Cu, Zn, Ni and Cr, while Gaskin et al. (2008) observed similar results in biochar produced from poultry litter. In regard to metals, feedstock seems to be the major determinant factor for their presence in biochar. With respect to PAHs, it is understood that they can be present in feedstocks but also be formed during the pyrolysis process, where their accumulation in biochar is dependent on both, their biomass source and the pyrolysis temperature at which that biomass is processed (Pakdel and Roy, 1991). It is generally recognized that temperatures >700°C are more likely to result in increased PAHs levels (Garcia-Perez, 2009).

1.3.2 Biochar' ageing: Consequences and possible effects

The stability of biochar is one of its main characteristics that make it such an interesting material from the perspectives of soil science, ecology and environmental management (Denef and Six, 2006, Lehmann et al., 2009). The recalcitrant behavior of biochar makes its application to soil a sensitive subject due to its heterogeneous nature and the complexity of interactions with soil structure and biota, and consequently, many soil processes and functions. As formerly referred, these interactions occur at the extensive surface area of biochar with a great cationic exchange capacity, where the numerous chemical functional groups react with the other components present of soil. Considering that soils themselves have a wide range of spatial and temporal variation in terms of composition, physical and biological properties and that in some cases, they are exposed to different environmental factors, it is therefore difficult to understand and predict safely what will be the impact of a particular biochar material. Given such heterogeneity both in biochar and in soil, the accurate measurement of its mean residence time in soil remains challenging (Brodowski et al., 2005, Brodowski et al., 2006), which added to the consequences of biochar ageing over time, are major knowledge gaps in biochar research (Verheijen et al., 2010).

Although predominantly stable, biochar is not inert and ages in soil over time, despite the specific mechanisms involved not being yet fully understood (Hammes and Schmidt, 2009). Nevertheless, some studies have shown evidence of factors that can contribute to biochar breakdown. While photochemical and microbiological activity seem to be the main cause of its degradation in natural soils (Goldberg, 1985) at slow pace, by modifying its surface area (e.g. pore blockage) and chemistry (e.g. loss of nutrient retention and CEC; Glaser et al. (2002). In human-altered environments, oxidation of biochar by chemical oxidants (Moreno-Castilla et al., 2000) and high temperatures (Cheng et al., 2006) can occur in the presence of microorganisms, at a faster rate (Zimmerman, 2010). Also, while ageing and oxidizing, biochar surface is thought to gradually become less hydrophobic and more charged, and as a consequence, content in carboxylic groups increase (Brodowski et al., 2005), changing the interaction capabilities (most times perhaps promoting further such interactions; (Cheng et al., 2006), with other soil organic, inorganic and biological matter (Brodowski et al., 2005), in which contaminants are included (Uchimiya et al., 2010). It is still unclear which are the biochar and environmental factors that most influence such changes in biochar properties (e.g. surface chemistry, particle and pore size and distribution as well as bioavailability of components) over time (Lehmann et al., 2009). Moreover, with progressive ageing accompanied by fragmentation into smaller particles, it is reasonable to expect a wider dispersion of smaller biochar particles in soils but also in water courses as time passes, which not only mean increase in biochar mobility (e.g. by soil erosion or bioturbation activity) but also in increased bioavailability of its contaminants (Cheng et al., 2006). Since fragmentation of biochar into smaller particles seems to be particularly important in boosting degradation speed, feedstock and pyrolysis conditions are key in defining the propensity of biochar to suffer fragmentation (Sohi et al., 2009). Likewise, is also reasonable that natural environments where phenomena such as cycles of freezing-defrosting, drying-wetting rain, higher level of bioturbation among others that promote fragmentation, also lead to an increase in biochar breakdown (Hammes and Schmidt, 2009). Such alterations can induce changes, yet difficult to predict, on its interactions with soil fauna and their activity, not only for the increase possibility of biochar ingestion by a range of soil organisms, but also in increased bioavailability of metals and PAHs in biochar (e.g. Hammes and Schmidt (2009); Wilcke (2000). In addition, Prodana (2011) showed that elutriates from biochar-soil mixtures obtained to simulate runoff or leachates from biochar-amended soils can induced toxic effects on aquatic organisms. Considering all these issues, it is important to continue research on the processes, mechanisms and implications behind biochar ageing in soil on the long-term, while further developing and standardising methods to simulate biochar ageing and mobility in soils.

1.3.3 Interactions between biochar with soil organisms

Despite some information already existing about the impact of biochar on the nutrient cycles, soil structure and microbial activity (Lehmann et al., 2003, Topoliantz and Ponge, 2003, Topoliantz and Ponge, 2005, Warnock et al., 2007, Downie et al., 2009, Van Zwieten et al., 2009, Grossman et al., 2010, Solaiman et al., 2010, Lehmann et al., 2011, Weyers and Spokas, 2011), there are yet several issues that need further investigation. Firstly, there is a great difference in researching of biochar effects on soil fauna and microorganisms, with the latter having received more attention due to their link to nutrient cycles, interaction with plants in the rhizosphere and thus, their role in agriculture and soil productivity.

Biochar impacts on soil fauna are yet to be understood. This is surprising since soil fauna plays a key role in ecosystem function by interacting with other soil organisms in the food chain and in maintaining soil structure, productivity and overall quality. Thus, their interaction with biochar might influence soil biological activity and/or the effect that biochar has on other biota populations (e.g. plants). Concerning effects on soil fauna, earthworms are the most well studied organisms. Nevertheless, consequences of biochar application to soil on this geophagous organism are far from understood, having both positive and negative effects being reported depending mainly on biochar composition and application rate. Whereas in some cases, earthworms have shown preference for biochar and even ingested it (Van Zwieten et al., 2009), in others case, they avoided it (Topoliantz and Ponge, 2003). Also, Noguera et al. (2010) found that inorganic-N increase greatly in soils amended with biochar, especially in the presence of earthworms, which has been thought to be related to the vertical mixing of biochar performed by earthworms during burrowing and/or their interactions with microorganisms (Major et al., 2010, Lehmann et al., 2011). Information about effects on other soil fauna, such as nematodes or microarthropods is yet very poor, with a scarcity of data that makes premature the taking of any conclusions, while studies in aquatic environments are even sparser (Prodana, 2011). Therefore, robust studies should be conducted on the long term, especially if we consider that the repercussions of biochar application to soil cannot be comparable to those of more labile and short-lived organic amendments, such as crop residues, compost or fertilizers, essentially due to the recalcitrant character of biochar. Moreover, since biochar can impact a wide range of soil processes mediated by biota, while interacting with other soil components and environmental parameters, it is crucial to understand the results and repercussions of its application to soils and even more so, when considering its long residence time in soil and the intention of possible large-scale applications worldwide, in the case of a carbon sequestration strategy. Therefore, whether we are studying the evaluation of biochar effects on plant productivity, on greenhouse gases emissions or on contaminant immobilization, these should always be accompanied by the impact assessment of biochar on soil organisms and finally on the interaction between biochar, soil, soil organisms (macro and microbiota) and contaminants, under different environmental conditions and varying abiotic factors (e.g. temperature, pH, humidity, light, etc.).

1.4 Ecotoxicological Bioassays

Since 2006, the Soil Directive (COM(2006) 232) was proposed to the European Parliament and of the Council by the European Commission aiming to increase protection of the soil environment, by cataloguing the contaminated sites inside EU territory and proceeding by a remediation through "actions on soil aimed to removal, control, containment or reduction of contaminants" with a controlled monitoring afterwards. In order to make an effective risk assessment of possible contaminated sites, this directive also establishes "the suitability of harmonising some of the elements of risk assessment as well as to further develop and improve the methodologies on eco-toxicological risk assessment". Considering the heterogeneity of biochar, its long-life in soil as well as the complexity of interactions that it can establish with soil mineral, organic and biological components, biochar ecotoxicology should too be methodically evaluated and for that, it is crucial to adapt and develop batteries of ecotoxicological tests to pin down the most suitable approaches for assessing the ecological risk of biochar.

In ecotoxicology, laboratory-based bioassays are carried out using standard tests with single species, due to their easiness, robustness, reproducibility and comparability of results between laboratories (Jensen and Pedersen, 2006). These ecotoxicological tests are fundamental to evaluate the risks of any substance in the environment, since they indicate the probable impact of those substances on one of the integral parts of the environment, its ecology. The same principle can be applied to biochar. Despite acknowledging their usefulness in risk assessment of many soil contaminants and their mixtures, the representativeness of such tests is poor in both aquatic and especially, in terrestrial systems, considering soil spatial heterogeneity and interactions of a multitude of organisms of different trophic levels and ecological function, and the way these are influenced by a variety of abiotic factors over time. In response to these disadvantages, tests carried out in soil mesocosms are useful for increasing the representativeness of the terrestrial ecosystem, accounting for more heterogeneity and variability, where a more realistic simulations of natural systems can be observed, while accounting for the interaction between organisms of different ecological function, if one wishes to. Mesocosms-based tests allow controlling a series of abiotic factors in the laboratory (e.g. moisture, temperature, photoperiod) but yet, because they are done in a greater scale including the amount of soil used (compared to the standard tests), the larger gradient in soil temperature, soil pH and moisture may difficult identifying which is the specific factor behind an observed effect (Burrows and Edwards, 2002, Edwards, 2002, Alonso et al., 2009). Small-scale Terrestrial Ecosystem Models (STEMs) are mesocosms that have already been used successfully for studying the ecological impact of various soil contaminants. For instance, STEMs have already proven to be effective in ecotoxicological evaluation of pesticide mixtures, as well the combined effects of chemicals and abiotic factors (e.g. Santos et al. (2011a)). This suggests that STEMs could also be suitable as a tool to assess the risks to soil ecology of a product so heterogeneous as biochar and, due to its setup, is suited for studying its implications on multiple species simultaneously (Santos et al., 2011b). The higher representativeness of this system will allow for more robust extrapolations of laboratory results to the field.

1.5 Test organisms

Earthworms

Eisenia andrei, a geophageous earthworm is one of the most well studied organisms in toxicology due to some particular qualities such as their sensitivity, abundance in most terrestrial ecosystems (high percentage of biomass among soil fauna), representativeness and role in important soil processes and functions (such as maintaining soil structure and aid in redistribution of organic matter), as well as easiness of keeping in cultures in laboratory (Luo et al., 1999, Lowe and Butt, 2007, Kim and Platt, 2008). In 1984, the European Union and OECD adopted them as standard organisms, while guidelines (ISO, 1993, 2005, 2012b) where published by the International Standard Organization regarding their suitability in toxicity testing. Endpoints evaluated usually include mortality, weight variation, reproduction rates and avoidance behavior.

Turnips

Brassica rapa is a turnip species, representative of the primary producers. *B. rapa* is sensitive to toxic elements, fast growing (short life cycles), bearing of seeds that have a high degree of homogeneity between them and that germinate after 3-4 days in soil. It is thus, suitable for toxicity tests, with specific tests having already been standardized (guideline ISO (2012a)). As a plant, it is quite easy to handle as well as to obtain their seeds. Germination rates and measure of both weight (fresh and dry) and shoot length are some of the most used evaluated endpoints (Stephenson et al., 1997).

Collembolans

Folsomia candida, a collembolan specie, is alongside *Eisenia andrei or E. fetida* maybe the most used soil organism in ecotoxicological tests, having already received attention from standardization organizations (ISO, 1999, OECD, 2009) and authors (Greenslade and Vaughan, 2003). The reasons that explain this resembles that of the earthworms, including widespread distribution, easiness to culture in laboratory and importance in soil ecology by being a major micro-decomposer, and as such playing a role in the food web (Fountain and Hopkin, 2005, Tully et al., 2006). The importance of these parthenogenic organisms is such that, their feeding behaviour influence microbial biomass and activity in soil (Kaneda and Kaneko, 2002). Therefore, toxic compounds that can affect collembolans, especially if soluble in soil pore water, food and air will affect their diet mainly consisting in bacteria and fungi (Crouau et al., 1999), causing deleterious consequences to the stability of soil as ecosystem .

Isopods

The interest of using arthropoda isopods, such as *Porcelionides pruinosus*, in ecotoxicology as a standard organism has been increasing due to their relevance as macro-decomposers on the litter fragmentation, meaning ultimately that their activity has repercussions on soil fertility by influencing nutrient cycling (Loureiro et al., 2006). However, standardized guidelines are not yet established for evaluating ecotoxicological responses of isopods to environmental contaminants (van Gestel, 2012), with Loureiro et al. (2005) proposing a behaviour (avoidance) test as a screening tool to assess soil contamination. Nevertheless, the difficulty in culturing isopods in laboratory where their aggregating behaviour by being cryptozoic organisms (Ferreira, 2009) restricts the evaluation of endeavour endpoints, being survival, growth and food consumption the most widely used (Drobne and Hopkin, 1994, van Gestel, 2012). Analysis on the activity and levels of relevant biomarkers such as acetylcholinesterase (AChE), lactate dehydrogenase (LDH), catalase (CAT), glutathione peroxidase (GPx), glutathione s-transferase (GST), lipid peroxidation (LPO) as well as energy reserves and cellular energy allocation (CEA) measurements when exposed to xenobiotics have also shown to be valuable tools for evaluating the effects of contaminants on isopods (Ferreira et al., 2010).

1.6 Research aim and objectives

Considering the lack of established methodologies for assessing the effects of biochar amended soils on soil biota and their performance, the main aim of this novel study was to adapt, optimize and test the suitability of a battery of simple, robust and low-cost ecological and ecotoxicological tools using representative soil organisms, to investigate the toxic potential of natural agricultural soils amended with biochar, at relevant application rates. To achieve this aim, the following objectives were met:

i) Testing the suitability of avoidance behaviour assays (using earthworms, collembolan and isopods) that are standardized or established in screening of contaminated soils, to assess the potential toxicity of soils enriched with wood-biochar, alone and in combination with traditional compost, over a 5 month period, in a real (on-going) field trial;

ii) Adapting and optimizing a methodology that uses Small-scale Terrestrial Ecosystem Models (STEMs) containing earthworms and plants, for higher-tier studying of the potential ecological impact of biochar on terrestrial ecosystems. In this case, a manure-biochar that has been considered as an alternative to fertilizers was selected, while the test set-up allowed accounting for longer study durations and greater heterogeneity in environmental conditions, in a way that can be more representative of natural systems.

1.7 Relevance of the study and applicability of results

So far, predicting the environmental risk of biochar application to soils has been done through chemical characterisation of biochar and biochar-amended soils. Having in consideration the overall aim of this research, it is therefore expected that results will fill gaps in current scientific knowledge on the toxicological effects of two different biochars (wood and manure) on an assembly of representative soil organisms and endpoints and on the suitability of already established (although for contaminated soils) tools to test such effects. Moreover, is expected that once tested the suitability of the developed tools, including the optimisation of STEMs methodology for assessing the risk of biochar application to soils, it can be an important contribution for complementing chemical characterization procedures of amended soils and for establishing a biochar risk assessment framework. It can also give relevant information in biochar characterization that can then be used to develop standards and certificates, as being proposed by the European Biochar Certificate (EBC) and the International Biochar Initiative (IBI). The interest of emerging biochar standards and certification schemes is a quality assurance, ultimately for environmental protection, as it guarantees a minimum set of physical and chemical characteristics so that biochar is safe for soil application. In this same context, it can help with optimizing the processing or treatment of biochars from organic wastes, to understand the suitability of this biomass source and safe application rates to soil. Throughout this study, the combined use of representative organisms that play an important role in the ecosystem but at the same time are sensitive to toxic compounds and variations in environmental conditions will provide more reliability to the results. It makes sense to think that only after adequate biochar risk assessment methodologies are developed and established, effective legislation can be developed for the use of biochar in agricultural fields.

1.8 Thesis structure and organization

The thesis is organized into four Chapters as described below, with Chapter II and III structured as an individual scientific paper.

- Chapter I: General Introduction, Research Aims and Relevance;
- Chapter II: Invertebrate avoidance behaviour as a screening tool for biochar-amended soils under viticulture
- Chapter III: Use of small-scale terrestrial ecosystem models for increasing environmental relevance in evaluating the toxic potential of biochar-enriched soils
- Chapter IV: Integrative Discussion, Concluding Remarks and Recommendations for Future Research.

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2 Bioassays Using Terrestrial Invertebrates Showed Suitability for Toxicity Assessment of Biochar–amended Soil in a Field Trial

2.0 Abstract

Biochar application to soils has been proposed as an important measure for increasing yield in dry lands, by reducing soil erosion and improving soil water and nutrient availability to plants. Portuguese viticulture is known to be particularly sensitive thorough stress, predicted by recent climate change models. The likelihood of using biochar application to soils under viticulture is currently being investigated in a field trial, as a strategy to help this sector in adapting and prospering under low water availability conditions. Nevertheless, before it can be applied in large scale, it is vital to assess possible toxicity to terrestrial organisms, which may lead to loss or deterioration of soil functions. With biochar ecotoxicology only now developing, studies focusing on biochar effects on terrestrial species remain scattered and lacking in environmental, ecological and practical relevance. This study used terrestrial invertebrate avoidance behaviour tests as a quick screening tool to evaluate the potential toxicity of pine wood biochar and a mixture of biochar-compost in a real field application scenario, over a 5 month period. The selected assays used earthworms (*Eisenia andrei*), collembolans (*Folsomia candida*) and isopods (*Porcellionides pruinosus*) exposed to soil (negative control), biochar (4 or 40 t/ha, positive control) and biochar-compost (40 t/ha, 4% biochar) for 48h, following well-established and/or standardized avoidance tests.

There was generally no avoidance of the tested organisms to either soil treatments, with isopods showing significant preference for fresh biochar and biochar-compost and earthworms avoiding the 5-months aged treatments, at the field rates. This suggests no short-term (fresh biochar applications) toxicity of pinewood biochar applied to a typical viticultural soil at field doses and highlights the need for long-term assessment. While no significant difference between collembolan avoidance behaviour responses were observed, isopod and earthworms were the most sensitive in differentiating between treatments and sampling times, at field rates. Data also suggest that terrestrial avoidance behaviour tests using invertebrates with different ecological functions may be an early, rapid and low-cost screening tool for toxicity assessment of biochar-amended soils in real field applications. Such an approach can be used for instance, to complement other strategies (e.g. physico-chemical characterization) in routine risk evaluation of biochar-enriched soils, in a way that is relevant for environmental or agricultural management.

2.1 Introduction

Low water availability and nutrient content which often leads to productivity decline in agricultural fields are due to a number of factors, from excessive and inadequate soil use and local geology-climate combinations to global climate change. This is a disconcerting subject considering the increasing needs of a growing population and has severe consequences that may further exacerbate the predicted climate change effects, including increasing risk in soil degradation and erosion. In Portugal specifically, viticulture and wine production is an important social-economic activity. Nevertheless, such activity is known to be strongly affected by climate and soil conditions, making it particularly sensitive to increasing drought stress, predicted by recent climate change models (Bridle and Pritchard, 2004). It is fundamental to develop strategies that can help agricultural soils, particularly the viticulture and viniculture sectors, to adapt to increasing low water availability conditions and maybe offer an opportunity to prosper under such a scenario.

Biochar is the carbon-based, heterogeneous and highly recalcitrant product of pyrolysis (300-1000ºC) of biomass, in the absence of oxygen. Biochar application to soils has been proposed as an important measure for increasing crop yield, by improving soil water and nutrient availability to plants while reducing soil erosion, during its long-mean residence time in soil. This is mostly due to its large specific surface area, highly chemically-reactive surface, neutral-to-alkaline pH (liming properties), high cation exchange capacity (CEC) and strongly aromatic structure, allowing for long-lasting complex interactions with soil physical, chemical and biological components (Lehmann et al., 2006, Spokas et al., 2009). The concept of biochar originated by thorough analysis of the fertile Amazonian black earths ("Terra Preta") soils, which owe their fertility and long-term stability to the combination of natural charcoal with mineral and organic wastes, including remains of human activity (e.g. pottery chards) (Glaser et al., 2001, Glaser and Birk, 2012). The application of biochar to viticultural soils, alone or combined with compost, as a strategy to help Portuguese viticulture to adapt and prosper under increasingly low water availability conditions is currently being tested in an on-going field trial at the Estação Vitivinícola da Bairrada (Anadia, Portugal). The combination of biochar and compost is expected to result in synergism for further augmenting soil quality and productivity, linked to improved water retention capacity, nutrient content and soil structure (e.g. porosity), enhanced microbial biomass and activity, as well as overall ecological function. Nevertheless, in order for large-scale biochar application to viticultural fields be sustainable, an assessment of the potential toxicity of amended soils on terrestrial organisms needs to be performed and adequate ecotoxicological and ecological tools need to be adapted and tested. With biochar ecotoxicology only now emerging and suitable standardized bioassays still lacking (Pakdel and Roy, 1991, Garcia-Perez, 2009, Verheijen et al., 2010), the likelihood of any toxic effects on terrestrial and aquatic organisms remain poorly understood, with plants and earthworms being used so far, almost exclusively (Lehmann et al., 2011). Further, while such studies remain often contradictory, scattered and lacking in environmental, ecological and practical relevance, the influence of biochar ageing in soils on its potential toxicity remain largely unknown (Verheijen et al., 2010). In its wider sense, 'ageing' refers to the series of processes that biochar goes through in soil leading to over-time alteration of its physical and chemical properties, which on the long-term can result in loss of stability, fragmentation into smaller particles, increased mobility through soils and enhanced bioavailability of biochar contaminants in amended soils as well as in fresh water systems (Moreno-Castilla et al., 2000, Wilcke, 2000, Glaser et al., 2002, Brodowski et al., 2005, Cheng et al., 2006, Hammes and Schmidt, 2009, Prodana, 2011) Bioassays are useful tools to assess the potential toxicity of environmental contaminants, since they focus on their bioavailable portion. Sub-lethal behavioural tests, such as avoidance behaviour response, have already proven effective as a simple and rapid screening tool in risk assessment of a range of soil contaminants and their mixtures (Jensen and Pedersen, 2006, Loureiro et al., 2005, Verheijen et al., 2012). Furthermore, the literature suggests that this endpoint is as much or even more sensitive than alternative sublethal endpoints, such as reproduction and growth, which demonstrates its reliability in environmental risk assessment applications (Da Luz et al., 2004, Loureiro et al., 2005, Natal-da-Luz et al., 2008, Natal-da-Luz et al., 2008) .It is based on the approach that the organism can prefer or avoid a treated soil, when given the option to choose between pristine soil and the soil containing the test compound.

This study used terrestrial invertebrate avoidance behaviour tests as a quick screening tool, with the aim to evaluate the potential toxicity of pinewood biochar and a mixture of biochar-compost in a real field application scenario, over a 5 month period. The selected assays employed earthworms (*Eisenia andrei*), collembolans (*Folsomia candida*) and isopods (*Porcellionides pruinosus*) exposed to soil (negative control), biochar (4 or 40 t/ha, positive control) and biochar-compost (40 t/ha, 4% biochar) for 48h, following established (in the case of isopods) and standardized (in the case of earthworms and collembolan) avoidance tests. The use of representative organisms and well-established methodologies increase reliability and comparability between studies. Considering their sensitivity to changes in soil conditions, as well as their relative role in the terrestrial ecosystem (e.g. in maintaining soil structure and stability if soil aggregates, nutrient recycling and redistribution, interspecific relationships with the remaining fauna), their combined use as test organisms will provide robust data on the potential toxicity associated to such field biochar application.

2.2. Materials and Methods

2.2.1 Field site, soil and biochar characteristics

The on-going field trial that lays context to this study takes place at the Estação Vitivinícola da Bairrada, part of the Regional Ministry of Agriculture (Direção Regional de Agricultura e Pescas do Centro- DRAPC), located in Anadia (Bairrada region of Portugal). The field supports 5 year-old vines of the cast Sauvignon Blanc, is open (no shade from buildings) and spatially homogeneous in terms of soil properties. The soil is a calcareous clay with the following main physico-chemical characteristics, expressed as mg/g (oven-dry soil): soil organic carbon (SOC), 12.1; water, 353; sand, 320; clay, 470; silt, 210; pH 6.36; bulk density 1.3 g/cm; with a predominantly Atlantic climate, annual average values of precipitation and temperature on site are 1000-1200 mm and 15°C respectively.

The test biochar and biochar-compost used in this experiment were obtained from the Delinat Institute - Swiss Biochar (Switzerland), where adequate control over the feedstock and pyrolysis as well as composting conditions ensure products with homogeneous physico-chemical properties. Biochar was produced from pyrolysis (620°C highest treatment temperature, 20 min residence time) of mixed wood residues from wood chip production. It’s following main physico-chemical properties allow classification of *premium grade* biochar (according to the supplier): density 552 kg/m^3; pH ($CaCl_2$) 10.1 (and 6.34, 6.73 and 6,89 when mixed with the test soil, at an application rate of 4t/ha, 40t/ha, and 40t/ha (with compost) respectively); moisture 30% (w/w), ash (550°C) 5 mg/kg, total C 75% (w/w), total N 1.8% (w/w); molar ratios (degree of aromaticity and maturation) H:C 0.074 and O:C 0.041; particle sizes: <0.1 mm (4%), 0.1-0.5 mm (25%), 0.5-2 mm (34%); >2 mm (37%); total contaminant contents: sum of metals (Pb, Cd, Cu, Ni, Hg, Zn, Cr, Bo) 171.27 mg/kg; sum of the 16 PAHs (US EPA) < 0.48 mg/kg; sum of the 7 indicator PCBs (dioxins) 0.00176 mg/kg. In relation to the biochar-compost mixture, its main properties included: biochar 4% (ww); moisture 36.4% (w/w); organic matter 38.7% among which, organic C 22.5% (w/w); mineral matter 24.9%; C:N ratio 18.4; N-NH_4 4.7 mg/kg; pH (CaCl2) 7.5; sum of metals (Pb, Cd, Cu, Ni, Hg, Zn) 165.7 mg/kg.

In the field, biochar and biochar-compost were incorporated manually into the topsoil (30 cm) of 4 m^2 field plots, in order to obtain the following treatments: soil (negative control plot), biochar (4 or 40 t/ha) and biochar-compost mixture (40 t/ha). The top 30 cm of the soil in each plot was removed using a spade, where the biochar was added at the treatment application rates and thoroughly mixed using a rake. The homogeneous soil-biochar mixture was then transferred back into the plot by spade where it was left to settle naturally.

2.2.2. Sample preparation and treatments

Due to restrictions (concerning interference with other field measurements) in taking amended soil samples straight after biochar incorporation, the samples corresponding to sampling time ‘0 months’ were fully prepared in the laboratory, using un-amended soil from the control plot and following the same procedure used in the field for the mixing of soil with biochar at two application rates (4 and 40 t/ha) plus a 50/50 mixture of biochar with compost (40 t/ha). For sampling time ‘5 months’, samples were collected from the field and brought into the laboratory in order to conduct the bioassays under controlled conditions. For all treatments and both sampling times, WHC of the soil was calculated though % water by mass using the equation:

$$\mathbf{u = [(m_{wet} - m_{dry}) / m_{dry}] * 100}$$

Where, **u** is water (%) by mass; $\mathbf{m_{wet}}$ is weight of saturated soil; m_{dry} is weight of dried soil (105ºC, 24 h).

For all treatments (soil, biochar and biochar + compost) at both sampling times, water content was adjusted to 60% WHC. The following abbreviations where used to distinguish between treatments, T0BL (Time 0, soil with biochar 4t/ha); T0BL (Time 0, soil with 4t/ha biochar); T0BH (Time 0, soil with 40t/ha biochar); T0BC (Time 0, soil with 40t/ha biochar + compost); T5BL (Time 5 months, soil with 4t/ha biochar); T5BH (Time 5 months, soil with 40t/ha biochar); T5BC (Time 5 months, soil with 40t/ha biochar + compost)

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2.2.3 Test organisms

The earthworm *Eisenia andrei* (Bouché 1972) used in this experiment was purchased from a commercial supplier, while isopod *Porcelionides pruinosus* (Brandt 1883) and the collembolans *Folsomia candida* (Willem) were obtained from cultures maintained in a climate chamber at 20 ± 2 ºC, 60% WHC and with a photoperiod of 16:8 (light:dark). The collembolans were kept in plastic boxes with a mixture of plaster of Paris as well as activated charcoal in a proportion of 9:1. Every week, granulate dried yeast was added in reasonable amounts on the two sides of the culture. Earthworms' individuals were more than one month old, with a developed clitellum and had a fresh weight between 250 and 600 mg.

2.2.4 Experimental setup and bioassays

Earthworms and collembolans avoidance tests were conducted following the respective ISO protocols (ISO, 2012a, ISO, 2011). Isopods avoidance tests were performed by adapting the procedure described by Loureiro et al. (2005).

To record the earthworm avoidance behavior, 10 earthworms were inserted in the center of a two section chamber with an area of ≈ 200 cm^2, divided vertically into two equal compartments. One contained 500 g of un-amended soil collected from the control field plot and the other, 500 g of biochar-amended soil at the study application rates. Exposure took place at 20°C and 16:8h photoperiod (light:dark) for 48 hours. Five replicates were used for each treatment.

Isopod avoidance behavior was recorded by inserting 5 specimens with antenna in the center of a two section chamber with an area of ≈ 135 cm^2, divided vertically into two equal compartments. One contained 50 g of un-amended soil collected from the control field plot and the other 50 g of biochar-amended soil at the study application rates. Exposure took place at 20°C and 16:8h photoperiod (light:dark) for 48 hours. Five replicates were used for each treatment.

For the collembolans assays, 20 specimens were inserted at the center of a two section chamber with an area of ≈ 57 cm^2, divided vertically into two equal compartments. One contained 30 g of un-amended soil taken from the control plot and the other 30 g of biochar-amended soil at the study application rates. Exposure took place at 20°C and 16:8h photoperiod (light:dark) for 48 hours. Five replicates were used for each treatment.

2.2.5 Statistical analysis

In order to calculate the avoidance behavior of organisms, while considering that organisms response will be dependent on their locomotion and distribution and assuming that organisms will avoid contaminated soils, the following equation proposed by Loureiro et al. (2005), was used:

$$\boldsymbol{B} = \frac{\boldsymbol{N} - 2^{*}\boldsymbol{T}}{\boldsymbol{N}} \; \mathbf{{*}100}$$

Where **B** corresponds to the organism’ behavior; **N** is the number of organisms per trial; and **T** is the number of organisms that were present in the treatment soil. For a statistical analysis of the effects of biochar on the organisms’ avoidance behavior, Fischer’s exact test was performed, using GraphPad’s scientific software, with a level of significance of $\alpha<0.05$ in all treatments for all tested species. In order to calculate AC50, a probit method was conducted using Priprobit (Sakuma, 1986). In addition, it will be used the recommendation from the ISO of considering a of avoidance as threshold. In this paper this point will be referred as "habitat function limit", as proposed by Loureiro et al. (2005).

2.3 Results

2.3.1. Earthworm avoidance behaviour response tests

The response of earthworms to the biochar and biochar-compost treatments was significant only for the five months period. A change from an initial small and non-significant preference to an avoidance at all concentrations was observed, with T5BL being significant, both when compared with control and between different sampling times (Fig. 1). It was however, not possible to calculate the effect concentration corresponding to 50% effect (AC50), even for the 5 month period.

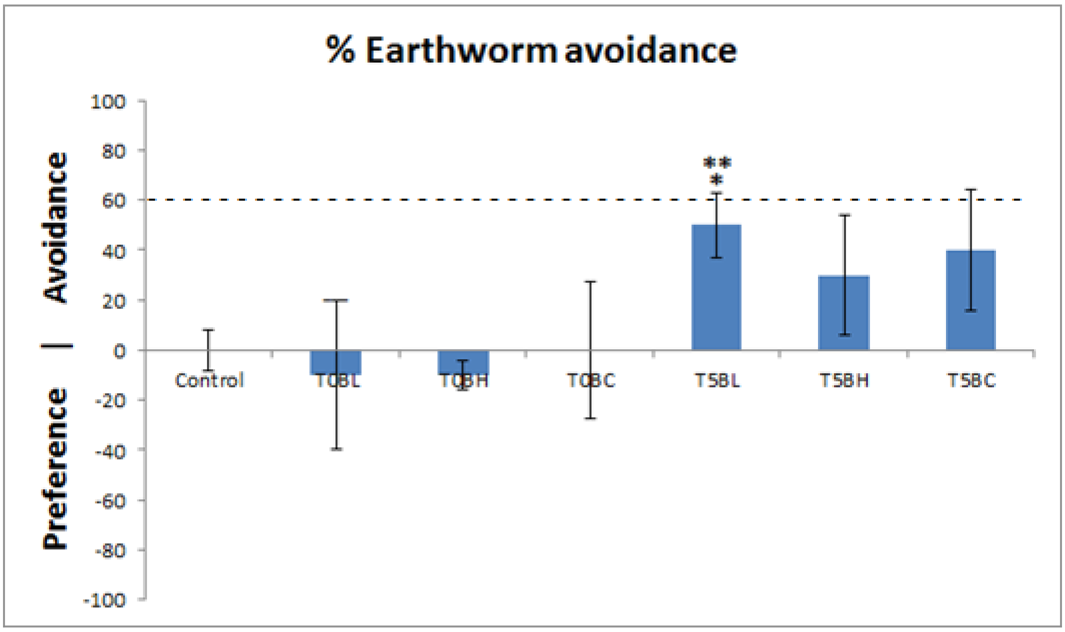

Fig. 1: Percentage of avoidance/preference of the earthworm *Eisenia andrei* upon exposure to the different soil treatments for 48 h. Treatments are: Ctrl (un-amended soil); T0BL (Time 0, soil with biochar 4t/ha); T0BH (Time 0, soil with 40t/ha biochar); T0BC (Time 0, soil with 40t/ha biochar + compost); T5BL (Time 5 months, soil with 4t/ha biochar); T5BH (Time 5 months, soil with 40t/ha biochar); T5BC (Time 5 months, soil with 40t/ha biochar+compost). Vertical bars represent standard errors of means of 5 replicates. The dash line states the ''habitat function limit''.*Indicates significant differences, Fischer Exact Test ($p<0.05$) compared to control;

**Indicates significant differences, Fischer Exact Test ($p<0.05$) in treatments for the time periods of 0 and 5 months.

2.3.2. Collembolan avoidance behaviour response tests

There was no statistically significant avoidance of the collembolan towards soil amended with biochar, both alone and in combination with compost. In fact, behaviour responses of collembolans towards the different treatments were not significant at any application rate (Fig. 2). However, organisms appeared to slightly avoid, without statistical significance, treatments at biochar applications rates of 4 and 40 t/ha, after 5 months. Due to the absence of significant differences it was not possible to calculate the AC50.

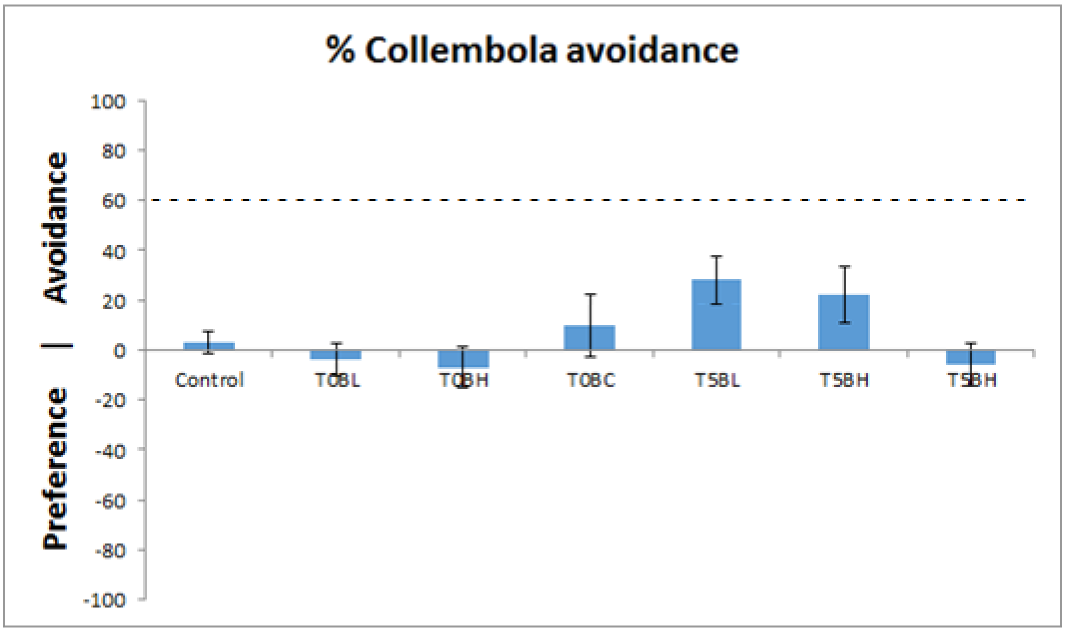

Fig. 2: Percentage of avoidance/preference of the collembolan *Folsomia candida* upon exposure to the different soil treatments for 48h. Treatments are: Ctrl (un-amended soil); T0BL (Time 0, soil with biochar 4t/ha); T0BH (Time 0, soil with 40t/ha biochar); T0BC (Time 0, soil with 40t/ha biochar + compost); T5BL (Time 5 months, soil with 4t/ha biochar); T5BH (Time 5 months, soil with 40t/ha biochar); T5BC (Time 5 months, soil with 40t/ha biochar+compost). Vertical bars represent standard errors of means of 5 replicates. The dash line states the ‘‘habitat function limit’’.*Indicates significant differences, Fischer Exact Test ($p<0.05$) compared to control;

**Indicates significant differences, Fischer Exact Test ($p<0.05$) in treatments for the time periods of 0 and 5 months.

2.3.3 Isopod avoidance behaviour response tests

Isopods' behaviour response indicated that there was preference towards biochar-amended soils, though only in treatments of 40 t/ha of fresh biochar there were significant statistical differences, with both T5BL (4t/ha) and T5BC (40t/ha of biochar-compost) being almost significant (P=0.0718) (Fig. 3). Whereas isopod's preference towards soils treated with 4 t/ha of biochar and with biochar-compost increased over time, that was less pronounced for 40t/ha – BH). Despite the dose-response pattern of preference observed to soils treated with fresh biochar, it was not possible to calculate an AC50 due to the high standard deviation at both sampling times.

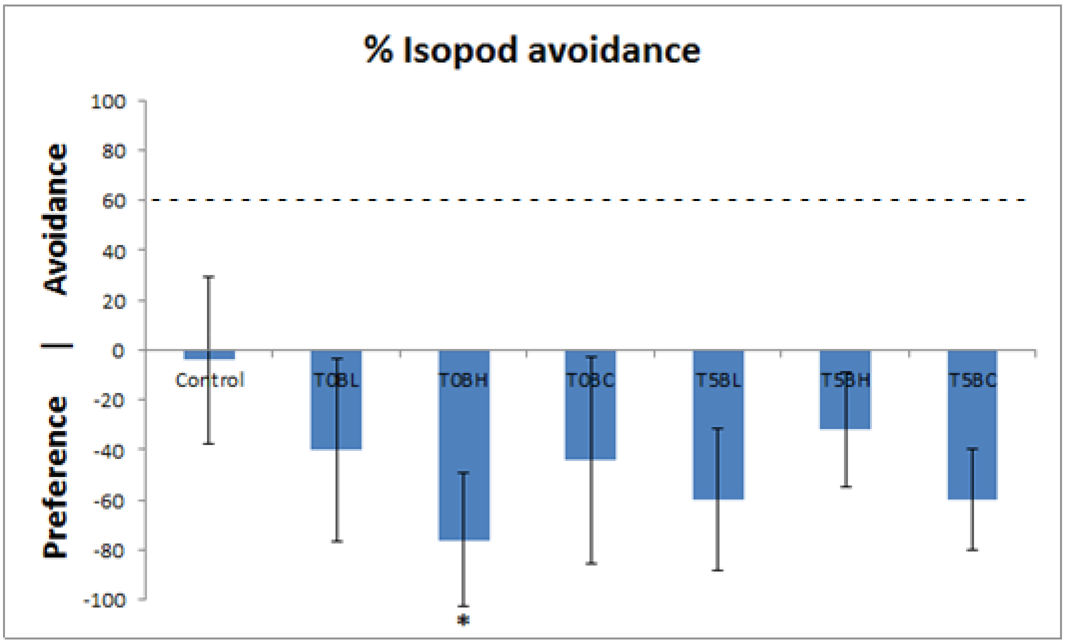

Fig. 3: Percentage of avoidance/preference of the isopod *Porcelionides pruinosus* upon exposure to the different soil treatments for 48h. Treatments are: Ctrl (un-amended soil); T0BL (Time 0, soil with biochar 4t/ha); T0BH (Time 0, soil with 40t/ha biochar); T0BC (Time 0, soil with 40t/ha biochar + compost); T5BL (Time 5 months, soil with 4t/ha biochar); T5BH (Time 5 months, soil with 40t/ha biochar); T5BC (Time 5 months, soil with 40t/ha biochar + compost). Vertical bars represent standard errors of means of 5 replicates. The dash line states the 20%

‘‘habitat function limit’’. * Indicates significant differences, Fischer Exact Test ($p < 0.05$) compared to control; ** Indicates significant differences, Fischer Exact Test ($p < 0.05$) in treatments for the time periods of 0 and 5 months.

2.4 Discussion

Behavioural responses varied according to the organism, when exposed to a typical viticultural soil amended with biochar alone and in combination with compost, at field rates of 4 and 40 t/ha, over the course of 5 months. Results suggest that on the short-term (fresh biochar applications), there is no significant avoidance of the tested organisms exposed to biochar alone and mixed with compost, with isopods exhibiting preference at the study conditions, when compared to the control. Nevertheless, on the longer-term (after 5 months), earthworms show significant avoidance for biochar alone, while for biochar-compost avoidance response was not significant. In contrast, there was no significant difference between collembolan avoidance behaviour responses between un-amended and amended soil, at such field applications rates. The test organisms were selected for their sensitivity to changes in their surrounding environment and for their role in main ecosystem processes and functions (e.g. in maintaining soil structure and stability if soil aggregates, nutrient recycling and redistribution, interspecific relationships with the remaining fauna), therefore linked to soil quality and to certain extent, to soil productivity. Combined, their responses provide a wider picture on the potential toxicity associated to such field biochar application.

It is possible that the reason for the observed differences in responses of the tested organisms may be predominantly due to their specific physiology, exposure pathways and properties of the biochar itself. The main routes of earthworm exposure to contaminants are skin (Reinecke et al., 1997, Jager et al., 2003, Vijver et al., 2003) and since they ingest of everything, the avoidance observed for the more aged biochar may be related to the release of contaminants not previously bioavailable. The very transformation of the surface of biochar, which is expected to be developed further by having more chemical groups, makes aged biochar more hydrophilic with increased probability to interactions with soil pore water that could lead to a large biochar fraction in soil solution (Moreno-Castilla et al., 2000, Wilcke, 2000, Glaser et al., 2002, Brodowski et al., 2005, Cheng et al., 2006, Hammes and Schmidt, 2009, Prodana, 2011). Also, perhaps at a smaller extent, some contribution from ingestion of small biochar particles due to biochar fragmentation could also have occurred.

Springtails have as their main route of exposure the contact with water (Ronday et al., 1997), although they can also be poisoned by the ventral tube (Lock and Janssen, 2003), although intoxication by feeding is not prevalent since these organisms can identify it due to the taste (Fountain and Hopkin, 2005). Thus, despite not as pronounced as in earthworms, it is possible that the avoidance observed to 5 month-aged biochar-amended soils (although not significant) may also be explained by biochar, although it is not clear a behavioural trend over time, there appears to be a decrease (although not significant) in the percentage of isopods' preference over time. This could mean contaminants becoming more bioavailable in soil solution as a result of fragmentation and/or increase in hydrophilic interactions. Nevertheless, considering the non-existence of significant differences in all treatments when compared to control, it is unclear if collembolan dispersion is a result of random behaviour or if there are indeed effects from biochar due to aging.

When compared to the two previous organisms, isopods have a more compact structure, and therefore the cutaneous poisoning route is more difficult, with the most probable pathway being the contact with litter, soil and water, the last of which not as important as in springtails. The preference observed by the isopods to soils with fresh biochar may have to do with the fact that soil became richer with nutrients, an observation that has been made by Gadd (2004). However a lower nutrient content with ageing (since the more labile nutrients would have already been used) or that increase in bioavailability of potential toxic substances could have lead to the slight reduction in isopod preference, particularly at higher biochar concentrations. The lack of a clear dose-response pattern for the isopods (ex: 4t/ha of aged biochar was statistical different but not 40t/ha of aged biochar (10x higher) and considering that they are known to be cryptozoic organisms, it is possible that statistical differences between treatments could be related to their common aggregation behaviour.

Results suggest that terrestrial avoidance behaviour tests using invertebrates with different ecological functions may be adequate as an early, rapid and low-cost screening tool for toxicity assessment of biochar-amended soils, in real field applications. Despite biochar application rates being on the lower end of the reported range (5 to 150 t/ha; Jeffery et al. (2011) the assays were sensitive in differentiating between un-amended and biochar amended soil as well as sampling times (to a higher or lesser extent depending on the species). There is therefore, potential for this tool to complementing other strategies in routine risk evaluation of biochar-enriched fields, such as chemical characterization and quantification of biochar and soil contaminants. Nonetheless, while the observed results overall suggest no short-term toxicity of pinewood biochar applied to a typical viticultural soil at field doses, longer-term toxicity assessment (>5 months) will be invaluable to conclude if the toxic potential of the biochar-amended field increases or decreases over time and on which organisms are more likely to be affected.

Further longer-term studies are necessary in order to test the sensitivity of the behaviour parameter for earthworms, collembolans and isopods to discriminate (with a clear dose-response pattern) between different biochar treatments and temporal differences, associated to biochar ageing. Also, avoidance behaviour responses could be complemented with other sub-lethal parameters, such as feeding behaviour, which could clarify whether effects are actually related to biochar ageing in soil or if they are only the result of random dispersion (e.g. in the case of collembolans) or aggregation behaviour (e.g. in the case of isopods).

2.5 Conclusion

Overall, there was no significant avoidance of the tested organisms to biochar alone or mixed with compost, suggesting no short-term (fresh biochar applications) toxicity of pinewood biochar applied to a typical viticultural soil at usual field doses. In fact, isopods exhibited preference for soil containing the fresh biochar, when compared to un-amended soil. Nevertheless, on the longer-term (after 5 months), earthworms show significant avoidance for biochar alone, while for biochar-compost avoidance response was not significant. Despite being not as sensitive (responses were generally not significant) as the remaining organisms to the amended soil, collembolans appear to show a slight avoidance behaviour at 5 months. Terrestrial avoidance behaviour tests using invertebrates with different ecological functions may be adequate as an early, rapid and low-cost screening tool for toxicity assessment of biochar-amended soils in real field applications. Such an approach can be used to complement other strategies in routine risk evaluation of biochar enriched soils, in a way that is relevant for environmental or agricultural management. Nonetheless, despite there was significant earthworm avoidance behaviour after 5 months, there is no clear dose-response pattern and thus, results are still inconclusive in relation to whether the toxic potential of the biochar-amended field increases over time. Further studies over longer term periods are necessary in order to test its sensitivity to temporal differences, associated to biochar ageing.

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3 Increasing Ecological Relevance in the Evaluation of the Toxic Potential of Biochar Enriched Soils

3.0 Abstract

The growing interest in large-scale biochar application to soils for improving soil properties and functions, sequestering carbon or replacing inorganic fertilizers, impels the development of effective approaches to evaluate its potential negative effects on soil biota and the functions that they facilitate. This work focused on adapting and optimizing the use of Small-Scale Terrestrial Ecosystem Models (STEMs) for assessing the potential toxicity of manure biochar (biochar-N) on the earthworm *Eisenia andrei* and the plant *Brassica rapa*. By comparing between responses of organisms with different ecological functions to biochar-N, in standardized single species tests and in STEMs, the overall aim was to understand if the use of STEMs is suitable for higher-tier evaluation of biochar ecological risks in soils.

Plant and earthworm performance when exposed to biochar-N was dependent on application rate, in both approaches (standardized and STEMs). However, the STEMs approach showed to be more conservative, with toxic effects observed at lower biochar-N concentrations (comparing to the corresponding standardized tests). Considering the increasing need for more representative testing conditions if ecotoxicological tools are to be routinely used in toxicity evaluation of real-case biochar applications, the use of STEMs that account for higher environmental and ecological variability, is suitable as higher-tier approach for assessing biochar's toxic potential to terrestrial ecosystems. Further, results can help in establishing biochar risk assessment procedures and highlight that application to soils of manure biochars should only be considered after identification and careful consideration of safe application rates.

3.1 Introduction

Biochar is the carbon-based product of pyrolysis (an exothermic process that occurs in the absence of oxygen) of biomass and has been recently object of an increasing interest in different fields of science and environmental management. Its application to agricultural soils has shown to result in improved crop yields while enhancing immobilisation of soil contaminants and contributing to combat climate change by sequestering and locking carbon in soil (Lehmann et al., 2006, Spokas et al., 2009). The observed agricultural benefits reside mostly in its large and highly reactive specific surface area, neutral-to-alkaline pH and a stable aromatic structure, which explains its long mean residence-times in soil. While its porous nature improves soil structure by increasing soil porosity and diminishing bulk density, it results in better water dynamics (e.g. retention or drainage), soil aeration, gas exchange and mobility of invertebrate organisms. Further, its high CEC and liming effect enhance the retention of nutrients and improves soil pH, and therefore augment microbial biomass and activity, which ultimately intensify biological performance of fauna and plant productivity (Schwartz et al., 2006, Atkinson et al., 2010, Compant et al., 2010, Graber et al., 2010).

Biochar production and use are also proposed to help in waste management, for instance, by adding value to abundant and cheap organic feedstocks, such as manures. Biochar produced from manure have a high content of nutrients (e.g. N, P, S) has been proposed as alternative to inorganic N-fertilizers, acting both as N source and sink, while reducing odor and ammonia emissions from soil (Day et al., 2005, Lehmann and Joseph, 2009, Schmidt, 2012). However, much uncertainty still exists regarding interactions between biochar and soil fauna as well on implications of any potential toxicity to biota, which can lead to loss or deterioration of soil functions. This is particularly true for manure biochars, considering that biowastes (e.g. animal manure, sewage sludge) can have higher levels of organic and inorganic contaminants(e.g. metals, PAHs, ammonia), compared to traditional biochar feedstocks (e.g. wood, crop residues; (Bridle and Pritchard, 2004, Gaskin et al., 2008). Until recently, only a few short term studies were performed in view of assessing biochar effects on soil organisms, with earthworm being that most commonly used. These studies are often contradictory, with some demonstrating preference of earthworms for biochar (Van Zwieten et al., 2009), while others shown high levels of mortality (Topoliantz and Ponge, 2003, Factura et al., 2010, Liesch et al., 2010), depending on a conjugation of factors, such as soil characteristics, heterogeneity of biochar, and their interactions (Bridle and Pritchard, 2004, Gaskin et al., 2008, Garcia-Perez, 2009, Verheijen et al., 2010, Verheijen et al., 2012).

Another important factor behind such discrepancy is the lack of established methodologies for assessing potential toxicity of biochar on terrestrial organisms, being particularly important the development and optimization of tool and approaches that have environmental and ecological relevance. Terrestrial bioassays using representative soil organisms are useful tools in environmental risk assessment of soil contaminants, because they respond only to the bioavailable portion of the contaminants in soil and thus, they add relevant information to chemical characterization of contaminated sites. In the same way, it is likely that they can be used for biochar risk assessment, with the same level of reliability. Despite standardized tests using single species being easy, robust, reproducible and allow comparing results between laboratories, they represent poorly the great environmental and ecological variability of natural systems. This is even more true in the case of biochar-amended soil, considering that biochar itself is highly heterogeneous and biochar-soil-biota interactions remain poorly understood.(Lehmann et al., 2011, Verheijen et al., 2010). Small-scale Terrestrial Ecosystem Models (STEMs) is a mesocosms model that has been used successfully for studying the ecological impact of pesticide mixtures in natural agricultural soil, since they add representativity and can account for greater environmental and ecological variation, such as larger moisture and temperature gradients, depth and the presence of another organism in order to include interactions between biological communities, as well as between them and soil conditions (Santos et al., 2011a, Santos et al., 2011b).

This work focused on adapting and optimizing the use of STEMs as a valuable tool for assessing the effects of manure biochar (biochar-N) on the earthworm *Eisenia andrei* and the plant *Brassica rapa*, in a way that is more representative of natural systems. By comparing between responses of organisms with different ecological functions to biochar-N, in single standard tests and in STEMs, the overall aim was to understand if the use of STEMs can be suitable for higher-tier evaluation of the ecological risks of biochar-N in soils.

3.2 Materials and Methods

3.2.1. Soil and biochar characteristics

The soil used in this experiment was natural agricultural topsoil (0-1 cm) with a loamy sand texture, collected from a pristine agricultural field located in the Mondego valley (Central Portugal), with no history of contamination or inputs of pesticides and inorganic fertilizers in the last 6 years. The soil had the following characteristics: sand 88.7%, clay 4.2%, silt 7%; pH 7.31; bulk density 2.4 g/cm^3; SOC 2.4%; WHC 70%; CEC 8.86 meq/100g. The soil was sieved (<2 mm) and air-dry (7 d, 20°C) prior to use.

The test biochar used in this experiment was obtained from the Delinat Institute (Switzerland) by mixing straw-rich manure, 10% grass clippings, 1% biochar (350-550ºC) and fermented with lactic bacteria (+ 3% vinasse). It has been developed for use as N-fertiliser with adjusted pH to avoid ammonia emissions (Schmidt, 2012). It's physical chemical characteristics were: pH 5.5 (biochar alone); dry matter 62% and moisture 38% (by gravimetry); 81.4% C; total Kjeldhal Nitrogen (org N + ammonia (NH_3-N) + ammonium (NH_4+-N) 17.1 mg N/Kg; particle size distribution (expressed as % weight) is shown in Table 1; water-extractable elements included Σmetals <70.37 µg/l (by ICP/AES-inductively coupled plasma-atomic emission spectroscopy) and Σ16PAHs <93.5 ng/l (by SPME-solid phase micro-extraction coupled to GC/MS-gas chromatography/mass spectrometry).

Table 1: Particle size distribution (PSD, in mm) of biochar-N, expressed as percentage.

PSD (mm)	Weight (g)	Percentage (%)
>4.00	74	37
0.50 - 2.00	68	34
0.125 - 0.50	50	25
0.50 - 0.125	8	4
Total	200	100

Soil water content was calculated based on mass of water per unit of mass of soil (using the following equation) and adjusted to 60% of its maximal water holding capacity (WHC):

$u = [(m_{wet} – m_{dry}) / m_{dry}]* 100$

Where, **u** is water (%) by mass; **m_{wet}** is weight of saturated soil; m_{dry} is weight of dried soil (105ºC, 24 h).

Biochar was added to soil at 5 application rates (10, 25, 50, 75, 100 t/ha) based on the reported application interval for maximizing crop productivity (Jeffery et al., 2011), after which WHC was adjusted to 60%. pH was measured for each soil-biochar treatment, as shown in Table 2.

Table 2: Comparison between initial and final pH values for the different treatment after biochar amendment.

Treatment	pH of un-amended soil	pH of biochar-amended soil
CTRL	7.2	6.45
BC10	7.2	6.93
BC25	7.2	7.34
BC50	7.2	8.30
BC75	7.2	8.61
BC100	7.2	8.86

3.2.2. Test organisms

The earthworm *Eisenia andrei* Bouché 1972 and the turnip *Brassica rapa* L. 1753 used in this experiment were obtained from a commercial supplier. Earthworms were more than one month old, with a developed clitellum and a fresh weight between 250 and 600 mg.

3.3. Experimental setup and bioassays

3.3.1. Single species exposure tests

Plant germination and earthworm avoidance tests (Fig. 4) were conducted following the respective ISO protocols (ISO, 2005, 2012a). Plant germination tests were performed using plastic pots (with a diameter of 90 mm) filled with 450 g of the test soil at

60% WHC (obtained through capillarity), where 10 seeds of *B. rapa* were sown at a depth of approximately 5 mm. Exposure was carried out at room temperature (20°C) with a photoperiod of 16:8h (light:dark) for 14 days (according to the guideline) and 28 days (adapted from the guideline). Endpoints recorded were plant germination rate (successful germinated seeds), fresh and dried weight and shoot length.

To record the earthworm avoidance behavior, 10 earthworms were inserted in the center of a two section chamber with an area of ≈ 200 cm^3, divided vertically into two equal compartments. One contained 500 g of un-amended soil (control) and the other 500 g of biochar amended soil at the study application rates and exposure took place at 20°C and 16:8h photoperiod (light:dark) for 48 hours. For both earthworm and plant tests, four replicates were used for each treatment including the control.

Fig. 4: Plant germination and earthworms avoidance behaviour tests, performed based on the corresponding ISO procedures 11269-2 and 17512-1.

3.3.2. Design and preparation of small-scale terrestrial ecosystem models (STEM)

To facilitate extrapolation of data to field conditions, an experiment was conducted in a controlled environment using STEMs, following the methodology described by Santos et al. (2011a), (2011b). Each STEM consisted of a cylindrical PVC pipe (12 cm diameter; 38 cm deep) sealed with a 1.0 mm thick plastic net at the bottom to prevent soil or earthworms from escaping, according to the schematic diagram in Fig. 5 A. The STEMs were inserted in acclimatized moveable carts (83 cm length; 55 cm width; 55 cm depth), each cart with the capacity for five STEMs. Each cart had automatic control of soil temperature (set to 12°C) (Fig. 5 B).

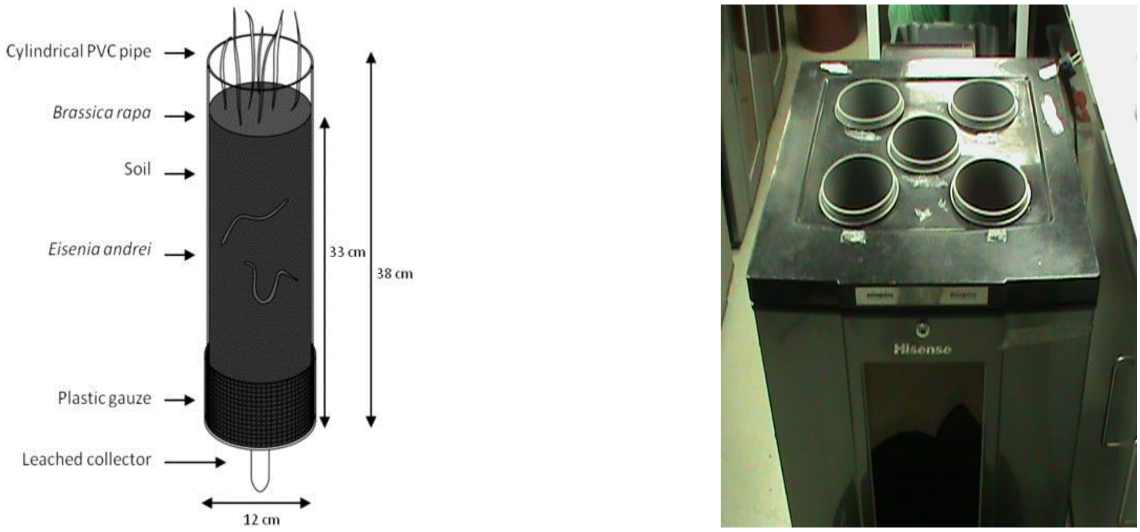

Fig. 5: (A) Schematic illustration of a Small-scale Terrestrial Ecosystem Model (STEM) used in this experiment (Santos et al., 2011a); (B) Acclimatized moveable cart containing five STEMs.

3.3.3. Experimental set-up in STEMs

In this experiment, 6 treatments of 0 (control), 10, 25, 50, 75 and 100 t/h of biochar at 3 replicates per treatment were used, in a total of 18 STEMs. The biochar was only applied into the top soil (0 - 15 cms) and the control soil was the lower layer (15 – 33 cms). In each STEM, 10 earthworms (weighing between 300-600 mg, with developed clitella) and 10 turnip seeds were introduced. The experiment ran for 28 days with a photoperiod regime of 16:8h (light:dark), at room temperature (20°C) and a soil temperature and moisture content of 12°C and 60% WHC. In order to maintain soil moisture content constant in the STEMs sterile water was added using a watering can, simulating rainfall conditions in Portugal's spring (≈ 85 mm per month, available at: http://www.weather-and-climate.com/average-monthly-precipitation-Rainfall,Porto, Portugal). The endpoints recorded were the same as for the standard single tests: plant germination success, fresh and dried weight and shoot length (for plants) and earthworm weight variation and distribution along the soil column (for earthworms).

3.3.4. Statistical analysis

Statistical analysis of the data obtained for all treatments in both single and mesocosms exposure tests were performed using Sigma Plot version 12.0 for operating system Windows 7. Analysis of variance (ANOVA) followed by post-hoc analysis Dunnet's was performed to test differences between treatment groups in STEMs and plant germination success rate in the single standard tests. To analyze differences in fresh weight and shoot length in plant single tests, a Dunn's analysis was carried out. To evaluate biochar effects on the behaviour of the earthworms in the single species exposure, it was conducted a Fischer test at a level of significance of $\alpha<0.05$. The calculations of EC50 were made using Priprobit (Sakuma, 1986) for the endpoints earthworm avoidance and germination rate in the standard single tests and earthworm distribution in the STEMs. For the parameter fresh weight and shoot length in the STEMs procedure, the EC50 was obtained using SIGMAPLOT v11.0 through a Logistic Model and Four Logistic Parameter Curve respectively.

3.4 Results

3.4.1 Responses to biochar in standardized single tests

Plant exposure to biochar-N caused an effect in all endpoints evaluated either for both 14 and 28 days period tests, with results expressed as percentage of the control (Fig. 6 and 7). In the 14 days test (Fig. 6) the fresh weight increased in the interval of 10 to 50 t/ha, and shoot length start decreasing at 50 t/ha. Germination progressively decreased in a dose-response manner, noticing significant differences starting at 50 t/ha. In the application rates of 75 and 100 t/ha there was no germination, and therefore all the endpoints were statistically different in those treatments. The pattern for the 28-day exposure was similar to the 14-day. While fresh weight (from 10 t/ha) and shoot length (from 25 t/ha) progressively increased with biochar application rate up to 50 t/ha, there was a decrease in germination rate from 50 to 100 t/ha, with no germination observed at 100 t/ha, corresponding to fresh weight and shoot length measures of zero (Fig. 7). The obtained EC50 values of 25.4, 19.6 and 73.6 t/ha for the fresh weight, shoot length and germination respectively (Table 3) are in line with these observations.

E. andrei avoidance behaviour when exposed to biochar-N was dependent on application rate in soil (Fig. 8), with earthworms preferring soils with biochar-N at 10 t/ha, while non-significant differences were found at 25 t/ha. Applications higher than 25 t/ha resulted in 100% avoidance, with an AC50 of 38 t/ha, as shown in Table 3.

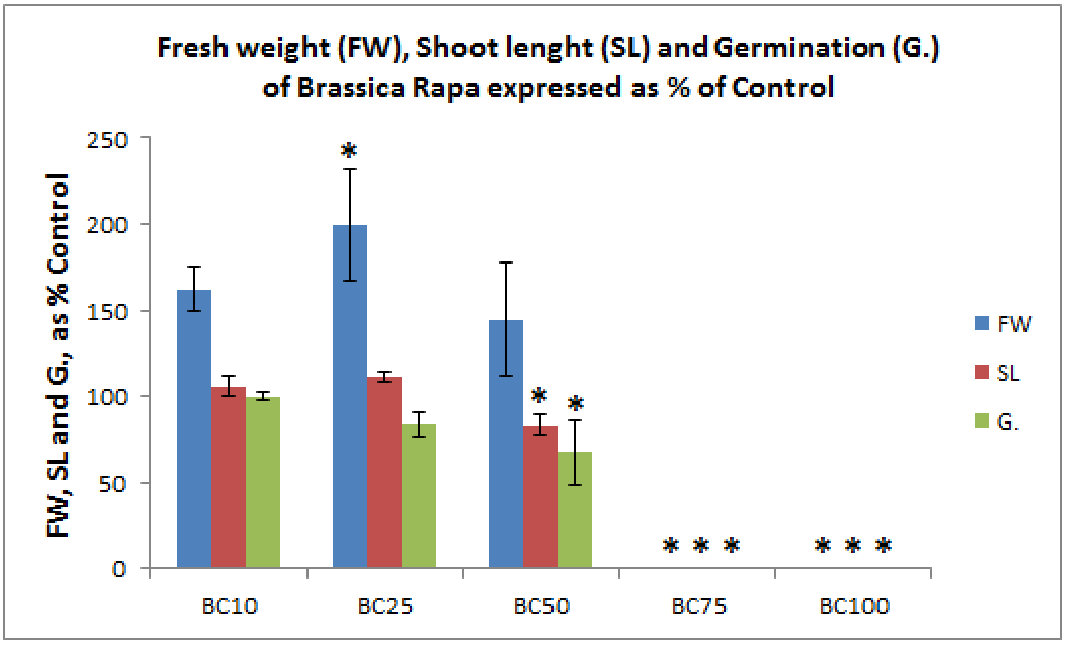

Fig. 6: Fresh weight (mg/plant), shot length (cm/plant) and germination rate of *Brassica rapa* expressed as % of control, when exposed to biochar-N (BC) for 14 d in standardized single tests. Treatment concentrations of 10, 25, 50, 75 and 100 are in t/ha soil. *Indicates statistical differences, Dunnett's method ($p < 0.05$) for FW, SL and G.

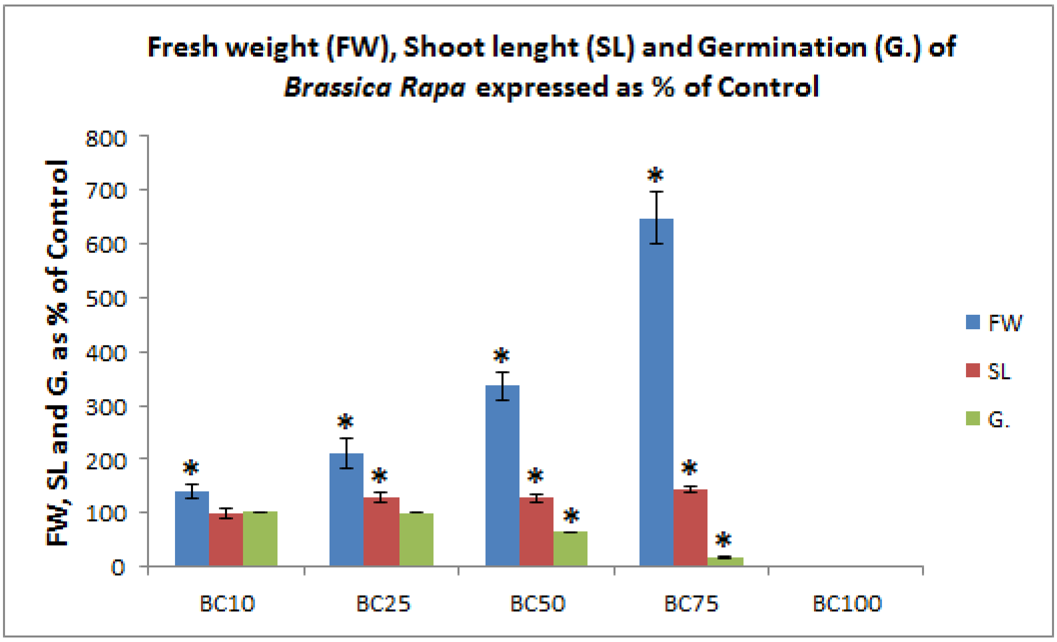

Fig. 7: Fresh weight (mg/plant), shot length (cm/plant) and germination rate of *Brassica rapa* expressed as % of control, when exposed to biochar-N (BC) for 28 d (adapted from standardized single tests). Treatment concentrations of 10, 25, 50, 75 and 100 are in t/ha soil. *Indicates statistical differences, Dunn´s Test ($p < 0.05$) for FW and SL and Dunnett's method ($p < 0.05$) for G.

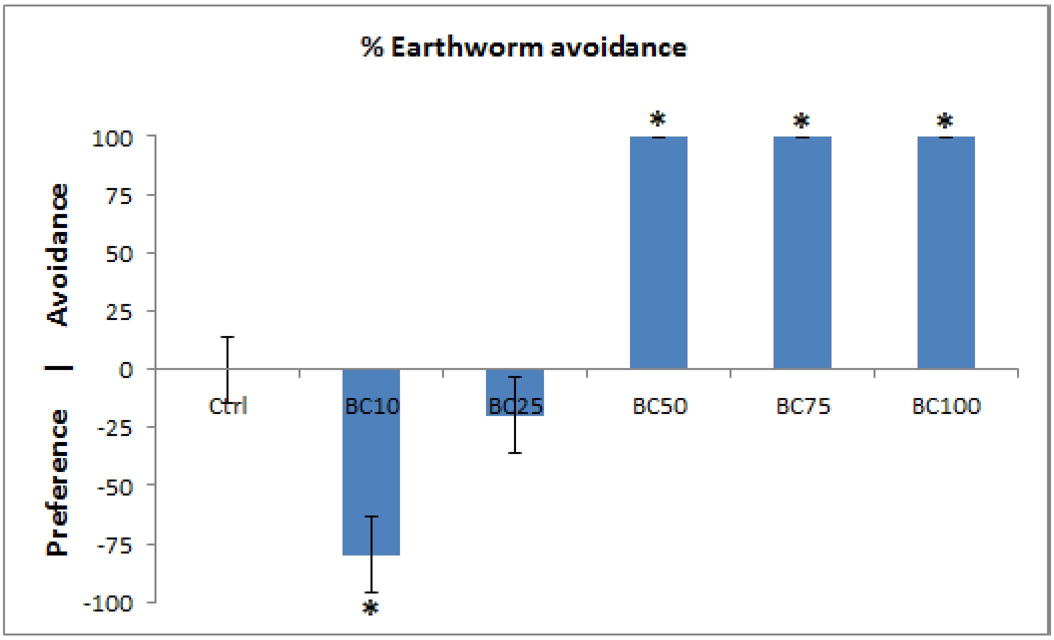

Fig. 8: Percentage of *Eisenia andrei* avoidance/preference when exposed to biochar-N (BC) for 48 h in standardized single tests. Treatment concentrations of 10, 25, 50, 75 and 100 are in t/ha soil. *Indicates significant differences, Fischer Exact Test ($p < 0.05$).

Table 3: Effect concentrations corresponding to 50% effect (EC50) and concentration corresponding to 50% avoidance (AC50) for plants and earthworms exposed to biochar amended soil for 28 days (for the plant parameters) and for 48 h (for earthworm avoidance behaviour) in single species tests.

Single Tests			
Plants EC50			Earthworms AC50
Fresh weight	Shoot length	Germination	Distribution
25.4 (16.4 – 34.5)	19.6 (2.6 – 36.6)	73.6 (68,4- 79,5)	38 (22.0 - 76.0)

3.4.2 Responses to biochar in STEMs

In STEMs, the evaluation of the endpoints for both organisms was done using the corresponding values for un-amended soil as reference. For plants (Fig. 9), while there was an observed increase in biomass and decrease in germination at 25 t/ha of biochar, compared to the control, there was a significant decrease in all measured parameters at, and above, 50 t/ha. Shoot length was either not significantly different from the control (up to 25 t/ha), or significantly lower than that (>50 t/ha). There was no seed germination in STEM for concentrations of biochar-N > 50 t/ha.

The distribution of earthworms along the STEMs' columns (with the control soil at the bottom layer of the column) followed a similar pattern as in the standardized single tests, although, contrary to observed avoidance starting at 50 t/ha (single tests), in STEMs it was observed for application rates of 25 t/ha, with avoidance however, being less prominent at higher concentrations (Fig. 10), which is in accordance with the EC50 obtained (Table 4). Soils treated with 10 t/ha of biochar-N have little effects on the distribution of earthworms, with a slight (although non-significant) increase in numbers on the first 15 cm of soil (biochar-amended soil), when compared to the control.

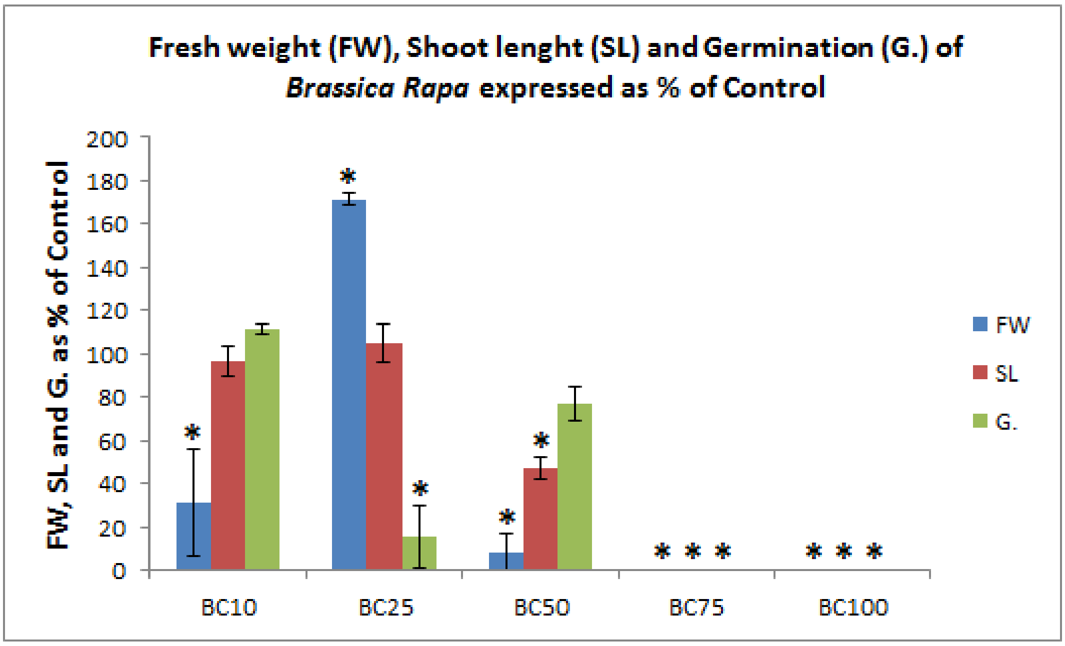

Fig. 9: Fresh weight (mg/plant), shot length (cm/plant) and germination rate of *Brassica rapa* expressed as % of control when exposed to biochar-N (BC) in the STEMs for 28 days. Treatment concentrations of 10, 25, 50, 75 and 100 are in t/ha soil. *Indicates statistical differences, Dunnett's method ($p < 0.05$).

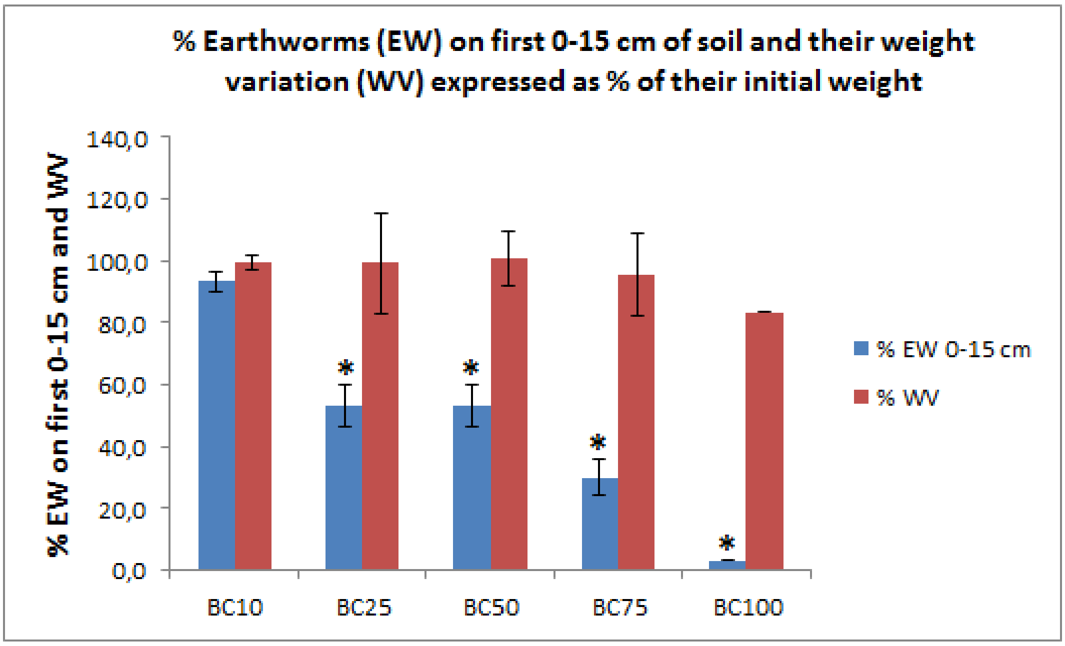

Fig. 10: Percentage of *Eisenia andrei* on first 0-15 cm of soil and their weight variation expressed as % of their initial weight when exposed to biochar-N (BC) in the STEMs for 28 days. Treatment concentrations of 10, 25, 50, 75 and 100 are in t/ha soil. *Indicates significant differences, Dunnett's method ($p < 0.05$).

Table 4: Effect concentrations corresponding to 50% effect (EC50) and concentration corresponding to 50% avoidance (AC50) for the plants parameters and earthworm avoidance behaviour, exposed to biochar amended soil for 28 days in STEMs. Not determined due to the absence of a solid pattern is indicate by 'n.d.'

STEMS			
Plants			Earthworms
EC50			AC50
Fresh weight	Shoot length	Germination	Distribution
n.d.	n.d.	n.d.	73.2 (71.1 - 75.3)

3.5 Discussion

3.5.1 Plant and earthworm responses to biochar-N

Considering the increasing need for more representative testing conditions in a way that can be routinely used in toxicity evaluation of real-case biochar applications, this work focused on adapting and optimizing a methodology based on STEMs and longer exposure conditions (up to 28 days), as higher-tier assessment of biochar's toxic potential to terrestrial ecosystems, when applied to soil at reported application rates (Jeffery et al., 2011). Looking at the results, it is understood that the performance of the organisms and the extent of response to the test biochar was different in both approaches. Using the standardized single species tests (14 days of exposure), plant endpoints indicate that the application of biochar-N to soil can enhance plant growth up to an application rate of 25 t/ha, but it starts to have negative effects on the germination rate from 50 t/ha, even though positive effects on the fresh weight and length of the plant are still observed. This may be related to biochar-N contribution with more nutrient (particularly N –being a limiting soil nutrient – and P) and water availability in soil as well as pore space for plant roots to grow, which results in less competition for resources, and consequently, an improvement in plant development. Thus, even though fresh weight and shoot length appear to be parameters demonstrating greater sensitivity, it is important bearing in mind that in this experiment, the germination rate decreases as the concentration of biochar increase in soil, and so the observed positive results in the other endpoints does not demonstrate an overall positive response. Therefore, this experience proved to be a good example of how the evaluation of several endpoints combined can be important. A possible explanation for the negative effects on plants observed at rates higher than 25 t/ha of biochar-N may be related with relatively high levels of ammonia (NH_3) in soil, which is corroborated by the increase in soil pH levels after 28 days (for both approaches, in the pots and in STEMs). *E. andrei* avoidance behaviour when exposed to biochar-N was also shown to be dependent on application rate, with earthworms significantly preferring soils with biochar-N at 10 t/ha, while applications higher than 25 t/ha resulted in 100% avoidance. It is likely that earthworm avoidance above 25 t/ha can be also explained by the ammonia levels. This, is not strange since ammonia accumulation in the terrestrial environment can compromise biological activity, with earthworms being especially sensitive to it (Hansen and Engelstad, 1999, Curry, 2004). Although the measured total Kjeldahl N after 28 days in biochar-amended soil (17.1 mg N/Kg) being within typical concentrations in soil containing fertilizers residues (> 10 mg N/Kg; Marx et al. (1996), considering the increase in pH from 7.2 to 8.86 (for the same time period) in the maximum application rate (100 t/ha), it is reasonable to suspect, to a certain extent, some contribution from NH_3 levels for the observed effects.

Plant and earthworm performance as response to biochar-N is in agreement with other observations. Factura et al. (2010) evaluated the potential of 'Terra Preta' Sanitation (TPS) and observed that earthworms (responsible for vermicomposting the TPS) died when exposed to charcoal and a lacto-fermented substrate, while Liesch et al. (2010) made similar observations in soils amended with poultry litter biochar. In both studies it was also suggested that the highly nitrogenous composition of the 'Terra Preta' and the litter biochar was the main cause for those results. An assumption was made from the observed increased pH values registered by Factura et al. (2010) and Liesch et al. (2010) over the course of the study, that ammonium (NH_4) salts present in the poultry manure would have become deprotonated generating ammonia (NH_3) during the test. On the other hand, PAHs and metal levels in the test biochar are below background soil concentrations, but also concentrations applied to the regulation of composted materials and EU regulations for sewage sludge application to agricultural soils (as reviewed by Freddo et al. (2012)), suggesting low environmental risk from their input in soil. Nevertheless, although PAHs and metals levels, as well as the pH itself, are not likely to be main cause for toxicity to the test organisms, it is possible that they could have contributed to the observed toxicity to a certain extent.

These results, instead of opposing to the use of biochar produced from N-rich biomass, such as manures, they highlight the necessity for a robust case-by-case evaluation of effects on representative organisms in order to, for instance, identify suitable application rates that will determine a safe application of manure biochars, but also, they may help in the monitorization and perhaps optimization of the efficacy of the post-treatment fermentation step. The use of acid-lactic fermentation as a post-treatment process in biochar made from these sources can be a valuable addition to the use of these products in a waste-management perspective, due to the fact that acid lactic bacterial activity results in lower ammonia levels because of its stabilizing effect on its precursor – urea (Schmidt, 2012).

3.5.2 Standardized single test vs. STEMs approach

By comparing the corresponding EC50 values between exposure in single species tests and STEMs, biochar-N has shown greater toxic in STEMs compared to single tests for both organisms. For the plants, while there was an observed increase in biomass and decrease in germination at 25 t/ha of biochar, compared to the control, there was a significant decrease in all measured parameters at, and above 50 t/ha. Fresh weight appeared to be the most sensitive endpoint, since differences were observed at the lowest concentration (10 t/ha). The relation between germination and fresh weight, (whereas germination rate increases as the fresh weight decreases or vice versa), did not allow calculating an EC50 value in STEMs. Therefore, from a practical point of view, it is also understood that after the turning point that marks the maximal productivity/application rate without negative effects on germination (25 t/ha on single tests and 0 t/ha on STEMs), there are two possible consequences: 1º) a decrease in shoot length and essentially in fresh weight, maintaining a germination rate similar to the control; or 2º) a low level of germination but with plants having increasing fresh weight. Furthermore, the same explanation that was given regarding single tests, where the availability of nutrients and water as well as space for root development might be related to the productivity, could be given to the STEMs' results.

In relation to the earthworms, their distribution along the STEMs' columns, which can be, although cautiously, referred to avoidance behaviour, was observed for application rates of 25 t/ha (while being less prominent at higher concentrations), thus, at lower biochar rates compared to that in single tests (> 50 t/ha). Although in STEMs, the vertical distribution behaviour of earthworms give us a better sense of the extent of stress of the organism, since such a distribution ('vertical avoidance') goes against the earthworm ecology. *E. andrei* is an epigeic earthworm (lives in topsoil) and while there were some individuals in the upper half of the column where biochar had been applied to soil, there was a clear preference for the bottom half of the column (control soil). Soils treated with 10 t/ha of biochar-N seemingly do not cause an adverse effect on this earthworm species, as a slight (non-significant) increase in earthworm numbers in the first 15 cm of soil was observed when compared to the control.

Differences between the two approaches can be explained by the larger variability in environmental and ecological conditions that is associated to the described STEMs approach, when compared to the standardized single species methodology. Specifically, there is a higher gradient in soil temperature (top vs. bottom of column) and soil moisture (particularly since irrigation was performed at the surface, when in the standard protocol is by capillarity), but also greater ecological relevance due to the possibility of accounting with the vertical distribution of earthworms and the biological interactions between both species. Differences in results, particularly in the case of earthworms, can also be explained by the longer study duration used in the STEMs approach (28 d) compared to the 48 h of exposure that is recommended for the standard assays. Longer exposure periods will likely to produce more robust results, because apart from the higher representativeness of the STEMs, temporal differences mean that the medium has become more complex, and more interactions occurred between biological, chemical and physical soil components. These factors that are not evaluated in the standardized tests can perhaps be determinant in the toxicity of biochar. It was in order to check the influence of the exposure time for plants, that the germination test guideline was adapted to 28 days also, which seemed to have been suitable for comparison with the methodology of STEMS, particularly since responses followed the same pattern observed for the 14 days standard germination test, although in 14 d test fresh weight seems to decrease after 25t/ha instead of increasing as in the 28 d test, thus the relation fresh weight/germination rate could explain this result. Furthermore, the presence of plants, due to the close interactions with earthworms, can also influence the behaviour of earthworms towards soils with biochar.

Overall, not only the STEMs approach is more representative of the natural environment but it also appears as a more conservative system, compared to that single standard tests. The higher conservatism in the STEMs is reflected in toxicity being observed at lower concentrations of biochar-N for all tested parameters and for both organisms, compared to the corresponding treatment in single tests, particularly for plants. From a practical point of view, it is also clear that, when looking at these results, the best application rates for plants (corresponding to higher benefits in yield) might not necessarily be the same for best earthworm performance.

Comparing responses of both organisms to biochar-N, *B. rapa* was maybe more sensitive than *E. andrei*, since toxic effects (fresh weight endpoint) started to be noted at lower concentrations of biochar-N. However, considering that *B. rapa* had two different negative response patterns towards biochar-N, in opposition to earthworms, the combined use of both species, each with a specific ecological function allowed increasing the level of confidence in the results. Also, considering the objective of this study, the selected biochar was a suitable choice, since it induced pronounced responses from earthworms and plants, over a range of biochar concentrations.

In summary, these results show that STEMs can be used for higher-tier toxicity assessment of soils amended with manure-based biochars, while it highlights that the application of biochars derived from organic wastes should be assessed case-by-case and based on previous identification and analysis of the most suitable concentrations, in order to be safe for application to soils. This observation comes to reinforce that proposed by (Verheijen et al., 2010, Verheijen et al., 2012), among other authors, while it also implies that for the farmer, there might need to be a balance and a compromise between the desired enhance in crop yield and what is acceptable for the remaining soil organisms. These results are expected to help in establishing appropriate methodology for toxicity assessment of biochar in soils, only after which, adequate legislation can be developed for biochar and biochar-N in particular. They can also contribute with relevant information in biochar characterization methods, as being presently a focus of the Food and Agriculture COST Action TD1107 on 'Biochar as option for sustainable resource management', European Biochar Certificate and the International Biochar Initiative (IBI).

3.6 Conclusions

Plant and earthworm performance as response to manure biochar is dependent on biochar-N application rate, in standardized single species tests and in STEMs, while toxicity to both organisms might be explained by biochar's ammonia levels, alone or in combination with other biochar contaminants. However, STEMs approach was more conservative compared to single standard tests, which is reflected in higher toxicity being observed at lower concentrations of biochar-N for all tested parameters and for both organisms. Considering the increasing need for more representative testing conditions in toxicity evaluation of biochar applications, the use of STEMs that account for higher environmental and ecological variability, as well as longer exposure conditions, is suitable as higher-tier approach for assessing biochar's toxic potential to terrestrial ecosystems. The selected manure biochar that is currently being considered as a possible alternative to inorganic fertilizers was a suitable choice for the objective of this work, while the combined use of representative organisms with different ecological functions allowed increasing the confidence level in the results. Results can be helpful in defining basic biochar requirements in standardization and certification and highlight that the application of biochars derived from manures should be assessed on a case-by-case basis, in order to be safe for application to soils.

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4 Integrative Discussion, Concluding Remarks, On-going Research and Recommendations for Future Studies

4.1 Integrative discussion and concluding remarks

It is becoming difficult to ignore the fact that biochar interest is increasing rapidly, which can be verified by the crescent number of articles on this subject (Verheijen et al., 2010). However, knowledge is still sparse in many areas, such as the effects on soil fauna and their functions. Most published studies address biochar's possible uses in agriculture (e.g. increased crop yields under low water availability conditions) and in environmental management and engineering (e.g. proposed role in carbon sequestration in soils and organic waste management). The analysis and understanding of the advantages appear greater than that of the potential harmful consequences, while it remains scarce the available information of biochar effects on terrestrial invertebrates and how that may influence the food chain and ecosystem stability and function. The contradictory information found in the current literature on the effects of biochar on soil organisms seem to some extent related to the lack of long-term studies in natural systems, and the absence of established methodologies that can take into account in an effective way the interactions of biochar with biotic and abiotic components in soil. Further, the heterogeneity of biochars (from the source material combined with the conditions of pyrolysis) and the variability in soil types and proposed application rates, make it difficult to compare between studies and extrapolate robust conclusions on this topic.

This study assessed the potential toxicity of two different biochars derived from widely available and cheap feedstock (pinewood waste from wood-chip production and manure-straw), presently being proposed for two specific applications, such as increased water retention in agricultural soils (alone or in combination with compost) and as a possible alternative to inorganic fertilizers (biochar-N), respectively. This was done by adapting and optimising methodologies (that are already established and successful in evaluating the risk of contaminated soils), to see if they are also suitable for risk assessment of biochar in soil and differentiate between control and biochar-amended soil and sampling times, as well as by analysing the combined responses of invertebrates representative of different ecological functions (E. andrei, B. rapa, F. candida and P. pruinosus) to amended soil treatments.

The first part of the work used terrestrial invertebrate avoidance behaviour tests as a possible quick screening tool to evaluate the potential toxicity of pine wood biochar and a mixture of biochar-compost on soil invertebrates (E. andrei, F. candida and P. pruinosus), under a real field application, over a 5 month period. Avoidance behaviour response assays have already proven suitable and valuable to assess the toxicity of soils contaminated with a range of single contaminants as well as their mixtures, including soils neighbouring an abandoned mine (e.g. Loureiro et al. (2005)). Data shows no avoidance of the tested organisms to fresh biochar or biochar-compost applications at field rates of 4 and 40 t/ha. Nevertheless, there was no significant difference between collembolan avoidance behaviour responses to the different treatments and sampling periods, at such field applications rates. While the observed results overall suggest no short-term toxicity of a wood biochar applied to a typical viticultural soil, results are not conclusive enough as to infer if the toxic potential of the biochar-amended field increases or decreases over a longer-time period.

Nonetheless, considering the need for more representative testing conditions, particularly if ecotoxicological tools are to be routinely used in toxicity evaluation of real-case biochar applications, the second part of this work focused on optimizing a methodology based on STEMs and longer exposure conditions (up to 28 days), as higher-tier assessment of biochar's toxic potential to terrestrial ecosystems. The STEMs methodology that was adapted and used had already been successful for toxicity evaluation of a mixture of pesticides in natural agricultural soil (Santos et al., 2011a, 2011b). The selected biochar in this case (biochar-N), produced from manure and currently being considered as a possible alternative to inorganic fertilizers (Schmidt, 2012) was a suitable choice for the objective of this work, since it induced pronounced responses from earthworms and plants, over a range of reported (Jeffery et al., 2011) biochar application rates. Besides adding larger environmental (e.g. soil moisture, temperature) and ecological variation (e.g. earthworm vertical distribution in soil, interactions among co-existing test organisms), the results using STEMs were also more conservative, showing toxicity at lower concentrations of biochar-N, when compared to the corresponding standardized single species tests. These results show that STEMs can be used for higher-tier toxicity assessment of soils amended with manure-based biochars, while it highlights that the application of biochars derived from organic wastes should be assessed on a case-by-case basis and only after identification of safe application rates, which can mean a compromise between what is ideal for crop production and for the remaining important biota. This observation comes to reinforce that proposed by Verheijen et al. (2010), among other authors.

Overall, outcomes of this study are robust and provide an overall picture and are therefore, expected to fill important knowledge gaps regarding the ecological risk of biochar-amended soils on terrestrial organisms, and provides preliminary information on the possible implications of biochar aging on representative invertebrates (this is still on-going work, see ‘On-going Research and Recommendations for Future work’). Presently, predicting biochar’s possible negative impact on soil processes and functions is mainly being done through physico-chemical characterization of pure biochar and mixed with soil, for example, in respect to quantification of total contents in contaminants, such as metals and PAHs (Hospido et al., 2005, Chan and Xu, 2009). This is reflected in the on-going work for standardization and certification of biochars, namely by the International Biochar Initiative and the European Biochar Certificate. With this study, it was showed that a range of ecotoxicological and ecological tools that are established for contaminated soils (and focused on bioavailable toxic components) can be used in combination to chemical characterization of biochars alone and mixed with soil, for evaluating their potential toxicity. This not only has practical use for biochar characterisation in real case applications, but also suggests that terrestrial assays could be maybe included in characterization procedures of standard biochars or even contribute to standardization of methodologies intended for evaluating ecotoxicological risks of biochar applied to soils. This is quite an important output due to the urgent need for standard biochar characterization methodologies as well as biochar certificates in view of safe use in a series of agronomical and environmental applications (e.g. increased crop yield, alternative to inorganic fertilizers, remediation of soil contaminants, carbon sequestration, etc). Studies like this that adapt and optimise existing methodologies for biochar risk evaluation show that it is possible to establish adequate and practical biochar risk evaluation procedures within the short-term, at low costs and with quick results (depending on the chosen endpoints), so that such an evaluation can be already possible as we speak.

Nonetheless, it is remains of course, necessary to keep improving the developed tools in a way that they can account for more and more environmental variation, such as changes during biochar ageing (see ‘On-going Research and Recommendations for Future work'). Also, further studies using other biochar types, application rates and complementary tests (e.g. testing chronic endpoints) are necessary to provide more information for a safe biochar application.

For instance, there is no doubt that biochar as an end to reduce or eliminate organic wastes and manures or sludges is very appealing. However, the fact that the feedstock may be rich in organic and inorganic contaminants, it is advisable to continue to develop and optimize the pyrolysis processes and any post-pyrolysis treatments as a way to act before, during and after the production of biochar, in order to significantly decrease the toxicity of the final product. Specifically, it is recommended to optimize the process of lactic fermentation in order to reduce the accumulation of ammonia, calibrate further the pyrolysis temperatures to minimize the production of PAHs and perhaps undertake other post-treatments of biochar, such as 'clean up' by incubating with selected microrganisms for degradation of biochar-bound PAHs or immobilization of biochar-bound metals using bacteria.

4.2 On-going research and recommendations for future work

Much of the work still in progress includes long-term assessing of the toxic effects of pinewood biochar and biochar-compost applied to the viticultural soil at the Estação Vitivinícola da Bairrada, which will enable studying the way the potential toxicity of the amended field changes over time, as influenced by biochar ageing in soil. A new field sampling is planned for January 2014, after 10 months of biochar application, followed by toxicity screening using the same battery of invertebrate avoidance behaviour response tests. It is expected that changes in the organisms responses over 10 months, alongside physico-chemical characterization of amended soil (which we are still expecting for 5 months and then, the 10 months) will clarify the invertebrate response pattern and allow linking changes in toxicity to a possible mechanism, such as changes in bioavailability of contaminants (e.g. metals and PAHs) in biochar-amended soil. To help in making such a link, it is being pondered conducting a series of established aquatic assays (e.g. bioluminescence inhibition of the marine bacteria Vibrio fischeri - Microtox®; growth inhibition of the microalgae Pseudokirchneriella subcapitata – OECD, 2006; and immobilization of the invertebrate Daphnia magna - OECD, 2004) based on exposure to elutriates of biochar-amended soil for the same sampling times. This is because soil elutriates not only simulate soil pore-water (being a major route of exposure to soil organisms), but also may give indication of any potential toxicity to aquatic ecosystems.

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